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Cadmium Accumulation and Distribution in Lettuce and Barley

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Abstract

Cadmium (Cd) is a non-essential trace element and its environmental concentrations are increasing due to human activities. Edible plants can accumulate high concentrations of Cd, which could be toxic to humans. Understanding how and where Cd is stored in plants is important for ensuring lower concentration of Cd in the food. In this thesis, the accumulation and distribution of Cd in three agricultural plants, namely lettuce (*Lactuca sativa* L.), barley (*Hordeum vulgare* L.) and radish (*Raphanus sativus* L.), were investigated with a focus on the potential mechanisms involved in the localization of Cd in the root. The main objectives of the study were: (1) to understand the effect of transpiration on Cd accumulation in lettuce, barley and radish, (2) to investigate the role of phytochelatins in Cd distribution in lettuce and barley, and (3) to determine the localization of Cd in the roots of lettuce and barley. The plants were grown hydroponically and analyzed using inductively coupled plasma atomic emission spectrometry, high performance liquid chromatography, and a combination of histochemistry and light microscopy, energy dispersive spectrometry, wavelength dispersive spectrometry, and x-ray fluorescence microscopy. The result showed that radish was sensitive to Cd and did not survive beyond 1.0 µM Cd. Below this concentration, radish accumulated negligible amounts of Cd in the edible organ (tap root) and was considered to have low risk of toxicity to consumers. Of the other species, barley accumulated more Cd in the root compared to lettuce, which was related to the ability of barley to retain more Cd in the root and possible redistribution of Cd from the shoot to the root via a phloem-mediated pathway. Barley provided more effective barriers against radial flow of Cd to the stele in the root and synthesized more phytochelatins and their precursor peptides in the root, which possibly immobilized Cd in the cytoplasm. Lettuce had most of its root Cd bound to the cell wall and the flow of Cd to the stele was less interrupted. This knowledge will be useful in designing or engineering plants with lower concentrations of Cd in the edible organs.

Keywords

Apoplast, cadmium, compartmentation, distribution, immobilization, phytochelatins, symplast, transpiration, uptake.

Co-Authorship Statement

The thesis includes three papers co-authored by myself, my supervisor and others. I am, or will be, the first author on each publication. The first paper entitled "Species-specific relationship between transpiration and cadmium translocation in lettuce, barley and radish" (Chapter two) is co-authored with Sheila M. Macfie (supervisor). The paper was published in the Journal of Plant Studies in 2012 (volume 1). While I designed and conducted the experiment, analyzed the data and wrote the manuscript, Dr. Macfie provided laboratory support, financial assistance and guidance in experimental design, data interpretation and manuscript preparation. The second paper entitled "Reduced translocation of Cd from roots is associated with increased production of phytochelatins and their precursors" (Chapter three) is co-authored with Brian McGarvey and Sheila M. Macfie, and has been submitted for publication. I designed and conducted the experiment, analyzed the data and wrote the manuscript. Dr. McGarvey provided some laboratory facilities, technical assistance in using HPLC and made useful comments about writing the manuscript. Dr. Macfie helped in designing the experiment, provided laboratory facilities, helped in analyzing the data and writing the manuscript. The third paper entitled "Distribution and chemical environment of cadmium in lettuce and barley roots by combining light microscopy, electron microscopy and micro x-ray spectroscopy" (Chapter four) is co-authored with Sheila M. Macfie and Desmond Moser. I designed and conducted the experiment, analyzed the data and am preparing the manuscript for publication. Dr. Macfie helped in designing the experiment, provided training and laboratory facilities for micro x-ray analysis, helped with data analysis and writing the manuscript and provided financial support throughout the study. Dr. Moser provided laboratory facilities and technical assistance in electron microscopy analysis.

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List of Abbreviations

ABA- abscisic acid

ACC- 1-aminocyclopropane-1-carboxylic acid

CAT- catalase

LMWOA- low molecular weight organic acid

MT- metallothionein

PC- phytochelatin

PCS- γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase)

POD- peroxidase

RH- relative humidity

RO- reverse osmosis

SE- standard error

SOD- superoxide dismutase

SRM- standard reference material

VPD-vapour pressure deficit

Chapter 1

1 General Introduction

1.1 Overview

Cadmium (Cd) is a naturally occurring trace element listed in group 12 of the periodic table of the elements. It has the greatest chemical similarity to the other elements present in group 12. The most common valence of Cd in natural environment is Cd (II) (Baes and Mesmer, 1976). In this thesis, Cd will be used to refer to cadmium in general, and Cd^{2+} will be used to refer specifically to ionic cadmium. Calcium (Ca^{2+}) has similar ionic radius and co-ordination pattern to that of Cd^{2+}, which can substitute for Ca^{2+} in the specific Ca^{2+} sites in phosphate minerals (Traina, 1999). Cd is a non-essential, potentially toxic element for both plants and animals. It is highly mobile and bioavailable in the environment (McLaughlin and Singh, 1999a).

Plants take up Cd^{2+} with water and nutrients when grown in Cd-contaminated soil. The amount of Cd taken up by an individual plant depends on the amount of bioavailable Cd present in the soil (Sheppard et al., 2007) and the physiological and morphological characteristics of the plant (Grant et al., 1999). Cd may accumulate in animals over time from ingestion of Cd-contaminated feed. Humans may accumulate Cd in their body through ingestion of plant- or animal-based foods and are at risk to develop chronic Cd toxicity (Dabeka and Mckenzie, 1992). Therefore, it is important to minimize Cd-toxicity in agricultural systems.

1.2 Cadmium in soils and plants

The major sources of Cd in soils are atmospheric emissions from mining and metal-using industries, direct application of phosphate fertilizers, sewage sludge, manure and composted municipal solid waste on agricultural soils, and accidental contamination from industrially contaminated land and mine waste dumps (Alloway and Steinnes, 1999). In Canada, phosphate fertilizers containing Cd are a major source of anthropogenic Cd in agricultural systems (Sheppard et al., 2007; Grant and Sheppard, 2008; Grant et al.,

2011). These fertilizers may contain Cd as a contaminant at levels ranging from trace amounts to as high as 340 mg/kg on a total dry weight basis, reflecting the concentration of Cd in the phosphate rocks from which the fertilizer was manufactured (Alloway and Steinnes, 1999). Long-term application of such fertilizers was reported to result in Cd-accumulation in agricultural soils in Canada (Sheppard et al., 2007), the United States (Mulla et al., 1980), Australia (Williams and David, 1976), New Zealand (Roberts et al., 1994), Britain (Nicholson et al., 1994), Norway (Baerug and Singh, 1990), and Denmark (Christensen and Tjell, 1991).

In order to be biologically relevant, Cd must be bioavailable to plants. Therefore, along with total Cd, the bioavailable fraction of Cd in the soil is also important in determining Cd toxicity to plants. Bioavailable forms of Cd in soil include free Cd^{2+}, Cd^{2+} complexed with organic ligands (Cd^{2+}-organic acids, Cd^{2+}-humate, etc.), Cd^{2+} complexed with inorganic ligands ($CdCl^{+}$, $CdOH^{+}$ etc.) and Cd^{2+} loosely bound to cation exchange sites in inorganic and organic soil particles. The ability to release Cd^{2+} from these complexes in the soil system depends on a number of factors including soil pH (Mann and Ritchie, 1993; Peijnenburg et al., 2000), organic matter (Murray et al., 2011), cation exchange capacity (Bolan et al., 2003a, 2003b), presence of chelators, e.g., organic acids (Cieśliński et al., 1998), presence of competing or complexing ions (Gao et al., 2011), and crop management practices (Gao et al., 2010).

The pathway of Cd^{2+} movement inside the plant is complex (Figure 1.1). Once available at the root surface, Cd^{2+} can enter the root apoplast (cell walls and intercellular spaces) through diffusion and mass flow. Inside the root, Cd^{2+} can bind either in the cell wall (Vázquez et al., 1992a; Wu et al., 2005; Vázquez et al., 2007; Wang et al., 2008) or pass through the plasma membrane using ZIP transporters (zinc (Zn)-regulated transporter/iron (Fe)-regulated transporter-like proteins; Cohen et al., 1998; Plaza et al., 2007; Pedas et al., 2008) or via cation channels, such as Ca^{2+} channels (Clemens et al., 1998). Other than via transporters or cation channels, Cd^{2+} can also enter the symplast (cytosol) by absorption due to the electrochemical potential difference between the activity of Cd^{2+} in the cytosol and that in the cell wall (Welch and Norvell, 1999). The difference arises from the activity of the H^{+}-translocating ATPase within the plasma

membrane that results in a large negative electric potential at the outer surface of the plasma membrane as well as very low activity of cytosolic Cd^{2+} compared to the apoplast (cell wall). Inside the symplast, Cd^{2+} can chelate with the peptides and organic acids present there and be sequestered in the vacuole (Rauser and Ackerley, 1987; Vázquez et al., 1992b; Liu and Kottke, 2004). Within the root cortex, Cd^{2+} can move through the apoplastic and/or symplastic pathways before it reaches the endodermis. The symplast is continuous from the epidermis to the vascular tissue because cells are connected by plasmodesmata. However, the Casperian strip in the endodermisblocks the radial flow of apoplastic Cd^{2+}; the only way across the endodermis is in the symplast. It is possible that Cd^{2+} can form complexes with peptides and organic acids present in the symplast of the endodermal cells and/or move towards vascular tissues using the plasmodesmatal pores (Clarkson et al., 1971). In the vascular tissues, Cd is translocated upward into the shoot with water as free Cd^{2+} or in complexed form with organic molecules present in the xylem sap. In the leaf, Cd moves from xylem into the leaf mesophyll cell apoplast from where Cd^{2+} can again be transported across a plasma membrane to enter the symplast. In order to enter seed or grains, or to be translocated back to the root, Cd^{2+} leaves the xylem and crosses the plasma membrane of a companion cell to enter the phloem sap for transport (Welch and Norvel, 1999).

1.3 Cadmium toxicity in plants

1.3.1 Plant growth

Cadmium (Cd) is considered a non-nutrient element for almost every living biota with the exception of *Thalassiosira weissflogii*, a marine diatom that uses Cd^{2+} as a substitute for Zn^{2+} to maintain optimal growth rate when Zn^{2+} is limiting (Lane et al., 2005). In most environmental conditions, Cd comes in contact with roots first and then moves towards other organs. The visible symptoms of Cd-toxicity in the roots include reduced root elongation (Aidid and Okamoto, 1993; Dong et al., 2005) and root browning (Arduini et al., 1994; Liu et al., 1995). Once Cd moves from the root to the shoot, leaf chlorosis and leaf rolling (Weigel and Jäger, 1980; Larsson et al., 1998) are the first visible symptoms to appear in the aboveground organs.

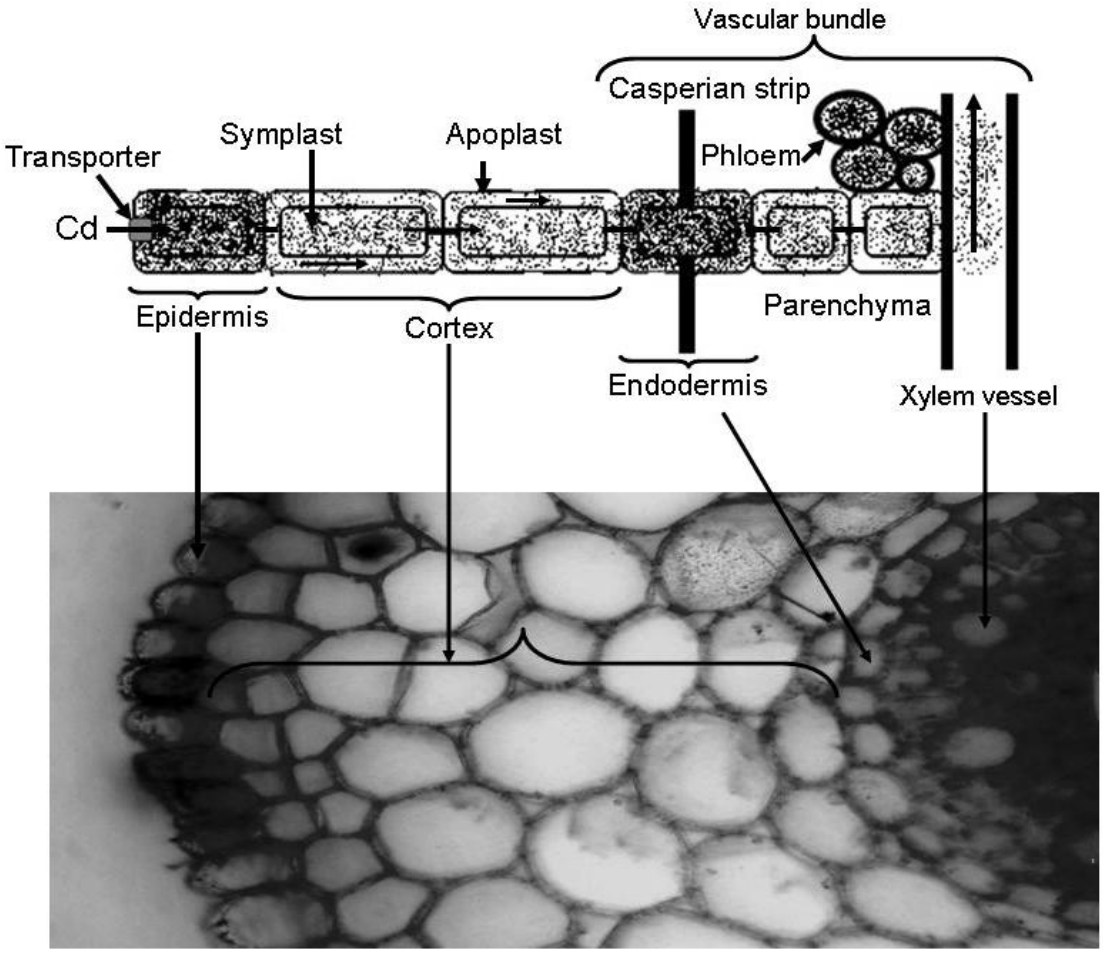

Figure 1.1: The potential pathway of Cd uptake and distribution in a root cross-section

Schematic representation (top) of a cross-section of a mature plant root (bottom) showing apoplastic (cell wall and intercellular) and symplastic (intracellular) pathways of Cd movement. Ions, including Cd^{2+}, can move in the apoplast as far as the endodermis, where they are blocked by the Casparian strip. Ions can enter the symplast through membrane transporters, and move from cell-to-cell via plasmodesmata. Ions are translocated to the aboveground tissues in the xylem, and may be translocated back to the roots in the phloem.

1.3.2 Nutrient uptake and plant-water relationship

Cd can affect the water balance in plants (Barceló et al., 1986; Poschenrieder et al., 1989; Costa and Morel, 1994) and can decrease the transpiration rate by inducing stomatal closure in the leaf (Haag-Kerwer et al., 1999; Vassilev et al., 2002; Mensah et al., 2008). Cd can inhibit nitrate reductase activity in the roots and can decrease the absorption and translocation of nitrate in plants (Petrovic et al., 1991; Hernandez et al., 1996). In *Glycine max*, 200 µM Cd^{2+} was also reported to damage nitrogen fixing nodules by inducing oxidative stress (Balestrasses et al., 2006), suppressing enzyme activity (Balestrasses et al., 2003) and increasing nodule senescence (Balestrasses et al., 2004). There have also been reports of Cd-induced Fe-deficiency in leaves of *Nicotiana tabacum* exposed to 100 µM Cd^{2+} (Yoshihara et al., 2006).

1.3.3 Photosynthesis and carbon assimilation

Accumulation of Cd can damage the photosynthetic apparatus. In *Raphanus sativus*, the function of light harvesting complex II was damaged in plants exposed to 0.2 mM Cd^{2+} (Krupa, 1988). Up to 50 µM Cd^{2+} was reported to damage photosystems I and II in *Phaseolus vulgaris* (Siedlecka and Krupa, 1996), and 5 µM Cd^{2+} decreased chlorophyll and carotenoid content in the leaf of *Brassica napus* (Larsson et al., 1998). Recently, proteomic analysis on multiprotein complexes from the thylakoid membrane showed that Cd induces a reduction in the antenna proteins of PSI; however, the effect was comparatively less in PSII and no changes were observed in the cytochrome b6/f and ATP-synthase complex organization (Fagioni et al., 2009). Other than the effects on photosynthesis, Cd was also assumed to decrease carbon assimilation in plants by reducing CO_2 uptake in the leaf (Perfus-Barbeoch et al., 2002).

1.3.4 Plant metabolic process

Although Cd^{2+} does not participate directly in cellular redox reactions (for example, Fenton reactions and Heber-Weiss reactions), exposure to Cd^{2+} can result in oxidative injuries, for example, enhanced lipid peroxidation in plants (Shaw et al., 1995; Gallego et al., 1996; Laspina et al., 2005). Gallego et al. (1996) found that 0.5 mM Cd^{2+} decreased the activities of ascorbate peroxidise, glutathione reductase, dehydroascorbate reductase

and catalase in *Helianthus annuus* . However, in *Phaseolus aureus*, 20 µM Cd^{2+} not only increased lipid peroxidation, but also the activities of guaiacol peroxidase, ascorbate peroxidase as well as catalase (Shaw, 1995). Similar findings were observed in *Phaseolus vulgaris* grown in 5 µM Cd^{2+}(Chaoui et al., 1997). Contrary to these studies, no lipid peroxidation was reported in the hairy roots of *Daucus carota* exposed to up to 1 mM Cd^{2+} (Sanità di Toppi, 1998). The reasons for contrasting results of Cd-induced oxidative stress in different plants could be due to the differences in the level of Cd to which the plants were exposed as well as differences in the concentration of thiol (-SH) groups, which can protect cells from oxidative damage, that were already present or induced by Cd^{2+} inside the cell.

Other than antioxidative ezymes, Cd^{2+} can also inhibit the activities of other enzymes in plants. For example, rubisco and carbonic anhydrase in *Phaseolus vulgaris* grown in up to 50 µM Cd^{2+} (Siedlecka et al., 1997), as well as glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, malic enzyme, and isocitrate dehydrogenase in *Silene italica* grown in 15 µM Cd^{2+} (Mattioni et al., 1997). Ju et al. (1997) exposed *Zea mays* seedlings to 20 µM Cd^{2+} and reported a marked increase in phosphoenolpyruvate carboxylase; however, they did not find further synthesis of glutamate dehydrogenase and glutamate synthase.

1.4 Plant responses to cadmium

1.4.1 Exclusion

Roots produce the first barrier against Cd-toxicity by secreting exudates. These exudates consist of sugars and organic acids that can bind Cd^{2+} present in the rhizosphere and hence make Cd less available to the plant. Costa et al. (1997) studied root exudates from *Lactuca sativa* and *Lupinus albus* seedlings exposed to Cd^{2+} and found that Cd^{2+} increased the concentrations of specific amino acids (asperagine, lysine and hydroxylysine) in the root exudates. A number of other studies also showed the ability of root exudates to bind metals other than Cd^{2+} in the root rhizosphere (Mench et al., 1988; Ma et al., 2001). Other than making Cd less available to the plant, root exudates also attract microorganisms (for example, mycorrhizal fungi) in the rhizosphere that can help

the plant to reduce Cd^{2+}-uptake. Mycorrhizal fungi form mutualistic associations with plant roots in which roots provide carbon to the fungi in the forms of root exudates and in return the fungi forms a hyphal network on the root surface and binds Cd^{2+} in the hyphae (Turnau et al., 1993), thus protecting roots from Cd $^{2+}$-exposure.

1.4.2 Immobilization

Once Cd^{2+} enters the root, the second barrier against Cd-toxicity is immobilization of Cd in the cell wall. The cell wall has pectic acids and hystidyl groups that can bind Cd^{2+} and make it less available to the metabolically active sites in the root. A number of studies have reported the cell wall to be an important site for binding Cd^{2+} at the cellular level (*Hordeum vulgare* grown in 5 µM Cd^{2+}, Wu et al., 2005; *Lupinus albus* grown in 150 µM Cd^{2+} for 35 d, Vázquez et al., 2007; *Bechmeria nivea* grown in 7 mM Cd^{2+} for 20 d, Wang et al., 2008). Contrary to these studies, other studies reported either absent or very low concentrations of Cd in the cell wall and most of the root Cd was either in the soluble fraction or in the vacuole (*Phaseolus vulgaris* grown in 0.45 mM Cd^{2+}, Weigel and Jäger, 1980;; *Agrostis gigantean* and *Zea mays* grown in 3.0 mmol/m^3 Cd^{2+}, Rauser and Ackerley, 1987; *Phaseolus vulgaris* grown in 0.5 µM Cd^{2+}, Vázquez et al., 1992; *Allium cepa* grown in 10 mM Cd^{2+}, Liu and Kottke, 2004). The varied results could be due to the differences between plant species and Cd^{2+} concentrations used in the studies as well as different Cd-detection methods used by various authors.

1.4.3 Compartmentalization

At the cellular level, Cd^{2+} can bind with thiol (-SH group) rich peptides and can store Cd in metabolically inactive sites, usually the vacuole. These specific peptides are called phytochelatins (PCs), a group of enzymatically synthesized peptides consisting of three amino acids: glutamic acid (Glu), cysteine (Cys) and glycine (Gly) (Grill et al., 1985). PCs are synthesized from glutathione by γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase, EC 2.3.2.15), forming the general structural formula of $(\gamma\text{-Glu-Cys})_n$-Gly, where n ranges from 2-11 (Grill et al., 1985; 1987; 1989). A number of metal ions were reported to be involved in the activation of PC synthase in plants, of which the strongest activation of the enzyme was observed with Cd^{2+} (Grill et al., 1989). The

activity of PC synthase is self-regulated, in that the product of the reaction (PC) chelates the enzyme-activating metal, thus terminating the enzyme reaction. Once PCs form complexes with metal ions they will either store the metal in metabolically inactivate sites inside the cell (Salt and Rauser, 1995) or release them to apoenzymes, which require these metal ions as cofactors to perform their catalytic activity (Grill et al., 1988). Phytochelatins are thus not only involved in metal detoxification, but also in metal homeostasis in plants.

Other than PCs, Cd^{2+}could also form complexes with organic acids present in the cytoplasm as well as in the vacuole (Sanità di Toppi and Gabbrielli, 1999). These complexes not only immobilize Cd inside the cell, but also prevent the circulation of Cd^{2+} in the cytosol.

1.4.4 Stress proteins

Plants produce specific proteins in response to Cd^{2+} inside the cell. These proteins are called stress proteins, and generally belong to the heat-shock protein (hsp) group. Cd-stressed cells produce specific mRNA transcripts that regulate the synthesis of stress proteins (Czarnecka et al., 1984; Edelman et al., 1988). The two most common Cd-stress proteins are hsp70 (Newmann et al., 1994; Reddy and Prasad, 1995) and its cognates (Reddy and Prasad, 1993) and ubiquitin (Jungman et al., 1993). Hsp 70 has strong affinity for abnormal proteins inside the cell and helps the plant by returning the misfolded proteins to their original forms (Jungman et al., 1993). Ubiquitins are highly conserved small hsps with only 76 amino acids (Hershko, 1988). They help the plant by degrading abnormal proteins in the cell that are produced due to Cd-stress (Jungman et al., 1993). Other than these proteins, Cd^{2+} also induces the production of several other proteins with molecular masses of 42,000 Da (Leita et al., 1991), 20,000, 22,000-24,000 and 50,000-65,000 Da (Delhaize et al., 1989; Urwin et al., 1996). Among these, the 20,000 Da proteins were found only in Cd-tolerant cells in *Datura innoxia* and are reported to belong in the stress proteins group (Urwin et al., 1996).

1.4.5 Stress ethylene

Plants produce ethylene in response to Cd-exposure. Initially, Cd^{2+} stimulates the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Furher, 1982a) and stimulates the synthesis of the ethylene precursor ACC, which can be converted to ethylene by ethylene forming enzymes (EFE, Burns and Evensen, 1986; Pennazio and Ruggero, 1992) in a MSAE pathway (methione-S adenosylmethionine-ACC-ethylene, Adams and Yang, 1979). Plants produce ethylene in an indirect way. At the cellular level, when Cd injures an individual cell, the cell sends a "mechanical injury" signal to the neighbouring cells and stimulates ethylene biosynthesis. At higher concentrations, Cd^{2+} decreases the activity of peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) and induces the accumulation of H_2O_2 inside the cell (Bhattacharjee, 1997). The accumulation of H_2O_2 induces lipid peroxidation and disrupts membrane integrity and the cell loses the ability to convert ACC to ethylene (Bhattacharjee and Mukherjee, 1996). Once ethylene production declines, the activity of soluble peroxidase increases and initiates the formation of insoluble phenolic material (lignin-like) in the cell wall surrounding the conducting tissues in the vascular bundle, which can reduce water and Cd translocation to the aboveground tissues (Furher et al., 1981; 1982b).

Ethylene might also have the ability to help the plant by regulating gene expression related to phytochelatins (PCs) and metallothioneins (MTs) (Sanità di Toppi and Gabbrielli, 1999), which can again help the plant by detoxifying Cd^{2+} at the subcellular level. As per knowledge, there has been no evidence supporting this assumption; however, ethylene is thought to bind its receptor through a metal cofactor, possibly zinc (Zn^{2+}) or copper (Cu^{2+}) (Ecker, 1995; Bleeker and Schaller, 1996). Since Cd^{2+} has chemical similarity to Zn^{2+}, it is possible that Cd^{2+} can replace Zn^{2+} and can alter (either increase or decrease) GSH metabolism and eventually affect PC synthesis.

1.5 Soil cadmium as a threat to human health

Cd has no beneficial effect on human health. The International Agency for Research on Cancer (IARC) has classified Cd as a human carcinogen (IARC, 1993) and it was also ranked number 7 in the Agency for Toxic Substances and Disease Registry (ATSDR)'s

1997 priority list of hazardous substances (Ostrowski et al., 1999). Once Cd enters the human body, it remains there for many years and may induce chronic toxicity. An exposure to higher levels of Cd is reported to cause itai-itai disease (Ogawa et al., 2004) and a combination of osteomalacia and osteoporosis (Ismail et al., 2002; Alfvén et al., 2004; Ogawa et al., 2004; Åkesson et al., 2006) in humans. Other effects include damage to the central nervous system, damage to the immune system, physiological disorder, cancer development, aggression and anxiety (Barański et al., 1983; Barański, 1986); however, most of these studies were performed on animals and confirmation is needed for the human health effects.

The main routes of Cd-exposure to humans are inhalation, ingestion and to a lesser extent by absorption through skin (McLaughlin and Singh, 1999b). Whereas inhalation is more related to occupational hazards, ingestion occurs through consuming Cd-contaminated foods. It was reported that 70% of the total Cd taken up by humans originates from plant-based food (Wagner, 1993). According to FAO/WHO (Food and Agriculture Organization of the United Nations/ World Health Organization), a provisional tolerable daily intake of 1 µg Cd/kg body weight is recommended. The average dietary intake of Cd by Canadians is 0.21 µg/kg/d (Dabeka and Mckenzie, 1992). Among everyday foods, fish, vegetables, bread and cereals contain the highest amount of Cd, and cereals alone can contribute up to 30 to 36% of the total dietary intake of Cd per day (Dabeka and Mckenzie, 1992). The commonly grown durum wheat in the Canadian prairies has an average of 0.28 mg Cd/kg grain (Garret et al., 1998), which is at or exceeding the CODEX Alimentarius Committee limit for cereal grains and oilseed traded on the international market (FAO/WHO, 2008). Therefore, along with potential health risks, it is possible that high Cd concentrations may hinder the exportability of grain and grain products.

1.6 Aim and outline of the thesis

Over the last few decades, scientists from various disciplines have been trying to understand the mechanisms of Cd- accumulation and how it is distributed among various plant tissues. However, it is still not clear where and how Cd^{2+} binds in the root before being transported to the shoot. In this thesis, I looked at the accumulation and distribution

of Cd in three agricultural plants, namely lettuce (*Lactuca sativa* L.), barley (*Hordeum vulgare* L.) and radish (*Raphanus sativus* L.), with a focus on the potential mechanisms involved in the localization of Cd in the root. The plants were chosen based on differences in morphological characteristics, and because they represent a leaf, grain and root crop, respectively. The main objectives of the study were: (1) to understand the effect of transpiration on Cd accumulation in lettuce, barley and radish, (2) to investigate the role of phytochelatins in Cd distribution in lettuce and barley, and (3) to determine the localization of Cd in the roots of lettuce and barley. The thesis consists of three manuscripts. The first, "Species-specific relationship between transpiration and cadmium translocation in lettuce, barley and radish" determined the effect of transpiration on Cd accumulation in lettuce, barley and radish (Chapter 2). A positive correlation between Cd content and total volume of water transpired was found in all the three species; however, the strength of the relationship was species-specific. The second, "Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors" examined the role of phytochelatins on the distribution of Cd in lettuce and barley (Chapter 3). Barley produced higher amounts of phytochelatins in the roots and was able to retain higher amounts of Cd in its roots compared to lettuce. The third, "Localization of cadmium in lettuce and barley roots by combining light microscopy, electron microscopy and micro x-ray spectroscopy" showed the cellular and subcellular localization of Cd in the roots of the studied species (Chapter 4). There were higher intensities of Cd in the dermal tissues of barley compared to lettuce and while most of the Cd was retained in the cell wall in lettuce, barley had equal distribution of Cd between the cell wall and inside the cell. Finally, the findings are summarized in two models in Chapter 5, in which the differences in Cd accumulation, distribution and translocation between lettuce and barley were discussed.

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Chapter 2

2 Species-Specific Relationship between Transpiration and Cadmium Translocation in Lettuce, Barley and Radish

2.1 Introduction

Cadmium (Cd) is a non-essential element for almost all biota with the exception of *Thalassiosira weissflogii*, a marine diatom that uses Cd^{2+} as a substitute for zinc (Zn^{2+}) in the metalloenzyme carbonic anhydrase (Lane et al., 2005). The two main sources of Cd in soils are geological parent materials and inputs from anthropogenic sources (Nriagu and Pacyna, 1988). Soils derived from Cd-rich parent materials can have concentrations up to 24 mg total Cd/kg (Alloway and Steinnes, 1999). Anthropogenic sources include the application of manure and sewage sludge as well as certain industrial activities. In Canada, Cd-contaminated phosphorus (P) fertilizers are one of the major sources of Cd-contamination in agricultural systems and concentrations of Cd in P fertilizers could be as much as 300 mg Cd kg^{-1} dry product (Grant and Sheppard, 2008). The mean Cd concentration in soil extracts can be as high as 0.17 µg/L, depending on the rate of P fertilizer application and the Cd concentration of the fertilizer (Lambert et al., 2007). Crops grown in contaminated soil may accumulate Cd in different plant parts, such as root, leaf, grain etc., and consumers may develop a number of Cd-related chronic diseases (Åkesson et al., 2006; Ogawa et al., 2004; Simmons et al., 2005). It is recommended that Cd concentrations be kept below regulatory guidelines in vegetables, fruits, grains and other agricultural products to avoid metal toxicity (Canadian Food Inspection Agency [CFIA], 2011). Because the concentration of Cd in edible plant tissues is not always directly proportional to the concentration of Cd in the soil (Wang et al., 2006; Hejcman et al., 2009; Smolders et al., 2009; Carbonell et al., 2011), understanding the mechanisms of Cd accumulation and translocation in plants is important to ensuring lower concentrations of Cd in food.

The ability of Cd to enter plants depends on a number of biotic and abiotic factors including plant species (Grant et al., 2008), microbial activity (Gao et al., 2010), soil pH (Mann and Ritchie, 1993; Peijnenburg et al., 2000), soil organic matter (Murray et al.,

2011), soil cation-exchange capacity (Bolan et al., 2003a, 2003b), presence of chelators, e.g., organic acids (Cieśliński et al., 1998), presence of competing or complexing ions (Gao et al., 2011), and amounts of total and plant-available Cd in the soil (Carbonell et al., 2011; Wang et al., 2006). Translocation of Cd within the plant depends on three major transport processes: passive and/or active uptake of Cd^{2+} into the root (Cataldo et al., 1983; Zhao et al., 2002), xylem transport from the roots to the shoots (Uraguchi et al., 2009), translocation to the seeds via phloem (Tanaka et al., 2007) and phloem-mediated redistribution of Cd from shoot to root (Van Belleghem et al., 2007). The first two processes are directly or indirectly correlated with water transport and transpiration rate. Since many forms of Cd are highly soluble in water, it is reasonable to expect a relationship between transpiration rate and Cd accumulation in plants.

The effect of Cd on transpiration of water from leaves has been studied extensively. At 10 µM Cd^{2+} and lower concentrations, Cd increased the permeability of the leaf cuticle and increased transpiration in sugar beet (*Beta vulgaris*; Greger and Johansson, 1992). At concentrations of 25 µM Cd^{2+} and higher, Cd induced stomatal closure and decreased leaf transpiration in mustard (*Brassica juncea*; Haag-Kerwer et al., 1999), barley (*Hordeum vulgare*; Vassilev et al., 2002), and lettuce (*Lactuca sativa*; Mensah et al., 2008). However, the mechanism of Cd-induced stomatal closure is still poorly understood. Some studies reported increased production of abscisic acid (ABA) with increased Cd-exposure and suggested that ABA might regulate stomata closure in Cd-stressed conditions (Hsu and Kao, 2003, 2005; Lòpez-Climent et al., 2011); however, in ABA-insensitive mutants of *Arabidopsis thaliana* Cd^{2+} affected guard cell regulation in an ABA-independent manner by entering the cytosol via Ca^{2+} channels (Perfus-Barbeoch et al., 2002).

While the effect of Cd on leaf transpiration has been well studied, little is known about the effect of transpiration on Cd accumulation and translocation in plants. In some cases, increased transpiration resulted in increased metal content. For example, when grown in artificial wastewater treated with different combinations of Cd and Zn ranging from 0 to 0.5 mM Cd^{2+}/Zn^{2+}, young wheat (*Triticum aestivum*) seedlings accumulated more Cd and Zn under conditions with high vapor pressure deficit (VPD) of the atmosphere compared to low VPD (Salah and Barrington, 2006). This finding is consistent with

populations of American pokeweed (*Phytolacca americana*) that showed a positive correlation between Cd accumulation and transpiration when grown in nutrient solution (Liu et al., 2010). In contrast, no relationship was found between transpiration and Cd concentration in shoots of inbred lines of maize (*Zea mays*) grown in the field (Florijn and Beusichem, 1993). The lack of consensus might be due to differences in species, duration of Cd exposure as well as the way transpiration was measured in the different studies. The species included hyperaccumulator weeds as well as low accumulator crop plants and the plants were either exposed to Cd in hydroponics for a short period of time or collected from contaminated fields. Transpiration measurement methods included amount of water lost per plant per day, amount of water lost per unit leaf area per second, and amount of water lost per unit dry weight of the shoot. The relationship between Cd content and these different measurements of transpiration may vary, especially if the plants being compared have markedly different leaf surface areas.

In this study, the hypothesis that the amount of Cd taken up and translocated to aboveground tissues is proportional to the total volume of water transpired in lettuce (*Lactuca sativa* L.), barley (*Hordeum vulgare* L.) and radish (*Raphanus sativus* L.) grown in a non-toxic Cd concentration was tested. These three species were chosen because of their broad range of leaf areas, which were expected to correspond to a range in volumes of water transpired per plant, and because they represent leaf, grain and root crops, respectively.

2.2 Methods and Materials

Chemicals, stock solution and reagents used were of analytical grade and all glassware was washed in soapy tap water, rinsed in tap water, soaked in 10% (v/v) hydrochloric acid (HCl) overnight, rinsed in RO (reverse osmosis) water and air-dried before use.

2.2.1 Germination and growth conditions

Seeds of each of three plant species, lettuce (*L. sativa* L. cv. Grand Rapids), barley (*H. vulgare* L. cv. CDC McGwire, hulless 2-row feed barley) and radish (*R. sativus* L. cv. Crimson Giant Champion), were germinated on moist (RO water) filter paper in Petri dishes in the dark for 24 hours. When the radicles were approximately 1 cm long,

seedlings were transferred to pots (15 cm diameter) filled with rinsed sand supplemented with nutrient solution (Table 2.1) adjusted to pH 6.0 using 1.0 mM HCl. The seedlings were kept in a growth chamber set to 21ºC with a 16 h light and 8 h dark cycle. The light intensity was 187 ± 2 μmol/m^2/s and relative humidity was set to 60%. After 7 days in sand culture, the roots were long enough to transfer the seedlings to hydroponics in 1.4 L glass jars. Different concentrations of Cd were added as $CdCl_2$ to the nutrient solution and pH was set to 6.0 using concentrated HCl before seedlings were transferred to the jars. In a preliminary experiment, it was determined that concentrations of Cd above 5.0 μM were toxic to lettuce and barley, and 1.0 μM Cd was toxic to radish. Therefore, the concentrations used in this experiment were 0, 0.10, 0.50, 1.0 and 2.0 μM Cd for lettuce and barley and 0, 0.05, 0.10, 0.20, and 0.50 μM Cd for radish. A total of three replicates were used for each treatment. In each jar (experimental replicate), one seedling was suspended in a folded 0.5 x 1 x 6 cm piece of foam and placed in a slot cut into a black plastic lid; this ensured that evaporative water loss was negligible. The sides of the jars were covered with black cloth to prevent algal growth. Each jar was hooked up to an aeration system and the plants were provided with fresh nutrient solution (including the corresponding Cd treatment) every second day.

2.2.2 Transpiration and growth record

The volume of nutrient solution lost per jar was determined by weighing the mass of each jar each time the nutrient solution was replaced. The daily transpirational water loss and the total volume of water lost were calculated from these values. The maximum amount of Cd that could be available through transpiration for each plant was calculated by multiplying the concentration of Cd in the nutrient solution by the total volume of water transpired. The maximum amount of Cd available for uptake was calculated by adding the mass amounts of Cd in each batch of nutrient solution provided to the plant. Plant growth was recorded as crown diameter (lettuce) or shoot height (barley and radish) and measuredon alternate days.

Table 2.1: Composition of nutrient solution

Macronutrient	Concentration (mM)	Micronutrient	Concentration (µM)
$Ca(NO_3)_2 \cdot 4H_2O$	1.0	H_3BO_3	6.0
K_2HPO_4	1.0	$MnCl_2 \cdot 4H_2O$	2.0
KNO_3	0.40	$ZnSO_4 \cdot 7H_2O$	0.50
$Mg(NO_3)_2 \cdot 6H_2O$	0.30	$CuSO_4 \cdot 5H_2O$	0.15
NH_4NO_3	0.30	Na_2MoO_4	0.10
K_2SO_4	0.10		
$FeCl_3 \cdot 6H_2O$	0.01		
Na_2EDTA	0.01		

2.2.3 Tissue harvest and biomass determination

Plants were harvested 28 days after Cd treatments were applied. At harvest, roots and shoots were separated, rinsed in RO water and blotted dry. Fresh weight and total leaf area (as measured using a LI-3100 leaf area meter, LI-COR Inc., Lincoln, Nebraska, USA) for each plant were recorded. The roots were rinsed in RO water for 30 seconds then placed in 1.0 mM $CaCl_2$ solution for 30 min followed by another 30 second wash in RO water (Taylor et al., 1998). This procedure desorbs Cd^{2+} from the root surface by means of a cation exchange reaction between Cd^{2+} and Ca^{2+} and would remove Cd-containing nutrient solution from the surface of the roots. All tissues were oven dried (60°C) until a constant weight was recorded.

2.2.4 Cadmium content

The concentration of Cd in roots and shoots was measured using a modified EPA test method SW-846 (United States Environmental Protection Agency [US EPA], 2005). The dried plant tissue was hand-chopped into fine pieces and ground using a mortar and pestle. A 0.1 g subsample was then placed in a 15 mL test tube and covered using a glass marble to prevent evaporation while allowing pressure to be released. Standard reference material (SRM) from the National Institute of Standards and Technology (NIST 1573a, tomato leaves) and reagent blanks were used to assess accuracy, quality assurance and quality control. All the test tubes were placed in a rack and 1.0 mL pure nitric acid (OmniTrace®, EM Science, USA) was added to each test tube to digest the organic matter. The samples were left overnight at room temperature. The following day, the test tube rack was placed in a shallow tray filled with sand and heated to 90-100°C on a hot plate until the vapors became transparent. Samples were allowed to cool to room temperature before being filtered (VWR, qualitative grade 413) into 50 mL sterile disposable centrifuge tubes. Reverse osmosis water was used to rinse the test tubes and bring the volume to 50 mL. The samples were analyzed for Cd content by inductivity-coupled plasma atomic emission spectrometry (ICP-AES) using the following conditions: Perkin-Elmer Optima 3300 Dual view ICP-AES, RF generator power -1300 Watts, plasma flow rate -15 L/min, auxiliary flow rate - 0.5 L/min , nebulizer flow rate - 0.8 L/min, pump flow rate - 1.0 L/min, analyte line - Cd 226.507 nm, plasma view -

axial, with a detection limit of 0.001 ppm for Cd. The percentage recovery of Cd in the digested SRM was 84 ± 5% and no Cd was detected in the reagent blanks.

2.2.5 Statistical analyses

SigmaPlot (version 11.0) was used for all statistical analyses and graphics. One-way ANOVA was used to detect treatment effects and Tukey's test was used to determine significant differences between treatment means (*P<0.05)*. Correlation and linear regression analyses were used to determine the relationship between transpiration rate and Cd content.

2.3 Results

2.3.1 Plant biomass

Cadmium treatments affected dry biomass of both the shoot and root tissues (Table 2.2). For lettuce, Cd exposure decreased both the shoot and root dry biomass and the leaves showed symptoms of Cd-induced stress (chlorosis, leaf rolling, etc.) at higher Cd doses. In the case of barley, Cd exposure slightly reduced shoot mass but had no effect on root mass (mid-panel in Table 2.2) and all seedlings looked healthy throughout the experimental period. Radish seedlings were sensitive to Cd and did not survive when grown in concentrations above 0.5 μM Cd. Increasing concentrations of Cd reduced shoot mass of radish and had no effect on their root mass (bottom panel of Table 2.2).

2.3.2 Plant transpiration

Total volumes of water transpired and water loss per unit leaf area varied with the Cd treatments (Table 2.3). Lettuce grown in the highest dose of Cd transpired 27% less total water than did plants grown in control solution, but transpiration per unit leaf area was 75% higher for plants grown with 2.0 μM Cd relative to control plants. For both barley and radish, plants grown at the highest dose of Cd transpired 36% less water volume as compared to control plants, and Cd did not affect transpiration per unit leaf area. Among the three species studied, radish transpired the largest volumes of water and lettuce transpired the least.

Table 2.2: Dry biomass (SE) of lettuce, barley and radish grown in different Cd treatments for 28 days

Treatments (μM Cd)	Shoot mass (g)		Root mass (g)	
Lettuce				
0	2.4 (0.3)[a]		0.6 (0.1)[a]	
0.1	3.6 (0.2)[a]		0.9 (0.1)[a]	
0.5	2.4 (0.2)[a]		0.7 (0.0)[a]	
1.0	2.1 (0.6)[a]		0.5 (0.2)[ab]	
2.0	1.0 (0.1)[b]		0.1 (0.0)[b]	
One-way ANOVA				
	$F_{(4, 14)}$	p	$F_{(4, 14)}$	P
	8.57	0.003	10.21	0.001
Barley				
0	1.8 (0.3)[a]		0.5 (0.1)[a]	
0.1	1.8 (0.1)[a]		0.5 (0.0)[a]	
0.5	1.1 (0.3)[b]		0.4 (0.1)[a]	
1.0	1.2 (0.2)[ab]		0.4 (0.1)[a]	
2.0	1.0 (0.1)[b]		0.4 (0.1)[a]	
One-way ANOVA				
	$F_{(4, 14)}$	p	$F_{(4, 14)}$	P
	3.86	0.038	1.15	0.387
	Shoot mass (g)		Lateral root mass (g)	Tap root mass (g)
Radish				
0	6.1 (1.1)[ab]		0.8 (0.1)[a]	5.2 (0.6)[a]
0.05	9.4 (1.2)[a]		1.0 (0.2)[a]	2.1 (0.7)[a]
0.1	4.6 (0.1)[ab]		0.6 (0.1)[a]	4.5 (0.4)[a]
0.2	3.4 (1.2)[b]		0.3 (0.1)[a]	2.9 (0.9)[a]
0.5	4.3 (1.1)[b]		0.6 (0.3)[a]	3.1 (0.7)[a]
One-way ANOVA				
	$F_{(4, 14)}$	p	$F_{(4, 14)}$ p	$F_{(4, 14)}$ P
	5.11	0.017	2.04 0.164	3.26 0.059

The results (F statistic and corresponding p value) of one-way analyses of variance for each tissue type within each species are also shown. Different lower case letters indicate significant differences in dry biomass, as determined by post-hoc tests.

Table 2.3: Total volume of water transpired (SE) and transpiration per unit leaf area (SE) by lettuce, barley and radish grown in different Cd treatments

Treatment (μM Cd)	Lettuce		Barley		Treatment (μM Cd)	Radish	
	Volume (mL)	Transpiration (mL/cm^2)	Volume (mL)	Transpiration (mL/cm^2)		Volume (mL)	Transpiration (mL/cm^2)
0	788 (94)^{a}	0.64 (0.05)^{c}	1095 (44)^{a}	1.74 (0.18)^{a}	0	3088 (19)^{a}	2.18 (0.44)^{ab}
0.1	957 (38)^{a}	0.51 (0.05)^{c}	1047 (12)^{a}	1.65 (0.06)^{a}	0.05	3138 (356)^{a}	1.34 (0.02)^{b}
0.5	731 (52)^{a}	0.59 (0.03)^{c}	683 (93)^{b}	2.02 (0.40)^{a}	0.1	2661 (156)^{ab}	2.3 (0.14)^{ab}
1.0	758 (98)^{a}	0.88 (0.04)^{b}	730 (67)^{b}	2.02 (0.09)^{a}	0.2	2187 (173)^{b}	2.1 (0.14)^{ab}
2.0	528 (54)^{b}	1.12 (0.05)^{a}	698 (32)^{b}	2.10 (0.28)^{a}	0.5	1971 (32)^{b}	2.5 (0.06)^{a}
$F_{(4,14)}$	4.53	31.88	12.60	0.73		7.52	4.06
p	0.024	0.001	0.001	0.59		0.005	0.033

The results of one-way analyses of variance (F statistic and corresponding p value) for each variable within each species are also shown. Different lower case letters indicate significant differences in transpiration per unit leaf area and total volume of water transpired, as determined by post-hoc Tukey tests.

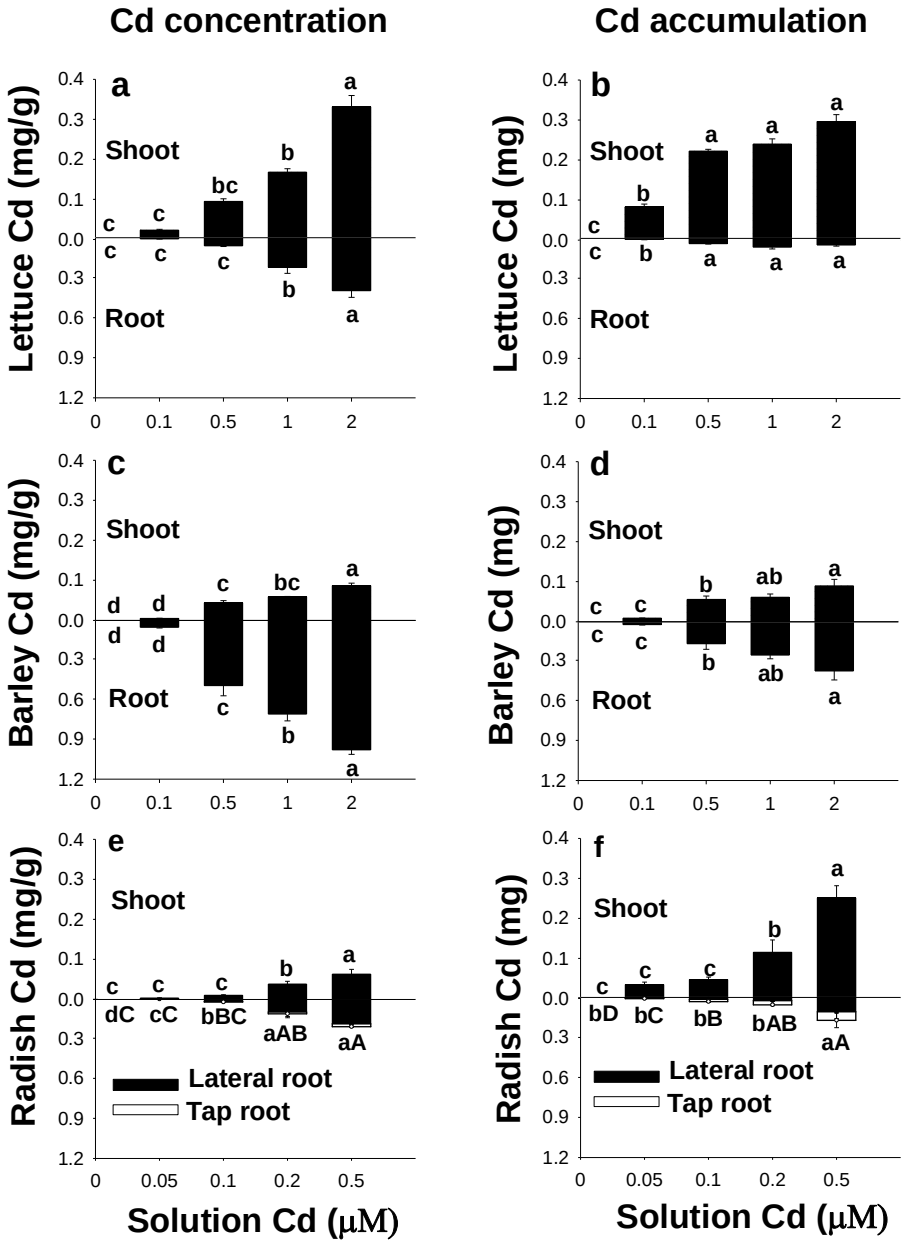

Figure 2.1: Concentration and accumulation of Cd in lettuce, barley and radish grown in different Cd treatments for 28 days

Concentrations of Cd (left-side panels) and total amounts of Cd (right-side panels) are shown for shoots and roots of (a,b) lettuce, (c,d) barley and (e,f) radish. Within each species, different lower case letters indicate significance differences in Cd concentration and Cd accumulation for shoots and roots, as determined by post-hoc Tukey tests. For radish, differences between lateral roots are indicated by lower case letters, and differences between tap roots are indicated by upper case letters.

2.3.3 Cd content

As expected, concentrations of Cd in the tissues of all three species increased as the concentrations of Cd in the growth medium increased. Among species, total Cd concentrations were highest in barley (Figure 2.1c) and lowest in radish (Figure 2.1e). Within species, shoot and root concentrations were equal under most experimental treatments for lettuce (Figure 2.1a) whereas Cd concentrations were higher in the roots compared to the shoots in barley (Figure 2.1c) and radish (Figure 2.1e). In radish, the lateral roots had much higher concentrations of Cd than did the tap roots (Figure 2.1e). When the total amount of Cd accumulated in each tissue was calculated (amount = Cd concentration × biomass) similar patterns emerged. The greatest amounts of Cd were measured in barley (Figure 2.1d) and the lowest amounts were measured in radish (Figure 2.1f). However, the three species responded differently in their ability to partition Cd among the different plant parts. Translocation of Cd from the roots to the shoots was measured by calculating shoot Cd as a percentage of total Cd (Table 2.4). In lettuce, 85% of the total Cd taken up by the plant was translocated to the leaves. In barley, most of the Cd taken up by the plant was retained in the roots and only 21% of the total Cd was translocated to the leaves. The pattern in radish was intermediate to the other two species; 66% of the total Cd was translocated to the leaves.

2.3.4 Solution Cd, transpiration and plant Cd

Regardless of the species, plants took up less than half of the total Cd supplied in the nutrient solution (Figure 2.2a) The total amount of Cd taken up by lettuce, barley and radish was approximately two to three times higher than the amount of Cd available through transpiration (Figure 2.2b), indicating the presence of other mechanisms, such as membrane transport, that facilitated the uptake of Cd^{2+}. The total amount of Cd in the plants was positively correlated with the amount of Cd available through transpiration (Figure 2.2b). The amounts of Cd translocated to the shoots of each species were also positively correlated with Cd available through transpiration (Figure 2.2c). However, the amounts of Cd in shoots of lettuce consistently exceeded the amounts predicted to be available through transpiration. Positive correlations were also found between the amount of Cd in the shoot and transpiration measured per unit leaf area in each species but the

Table 2.4: Shoot Cd as a percentage of total Cd (SE) in lettuce, barley and radish

Species	Proportion of total Cd in the shoot (%)	
Lettuce	84 (2)[a]	
Barley	21 (1)[c]	
Radish	66 (3)[b]	
One-way ANOVA		
	$F_{(2,35)}$	p
	296.41	0.001

Data from each Cd treatment (0.1-2.0 µM Cd for lettuce and barley, and 0.05-0.5 µM Cd from radish) were pooled (n=12 for each mean value) prior to the calculation of the proportion of total Cd that accumulated in the shoot. The results of the one-way analysis of variance (F statistic and corresponding p value) are also shown. Different lower case letters indicate significance differences in Cd translocation, as determined by post-hoc Tukey's tests.

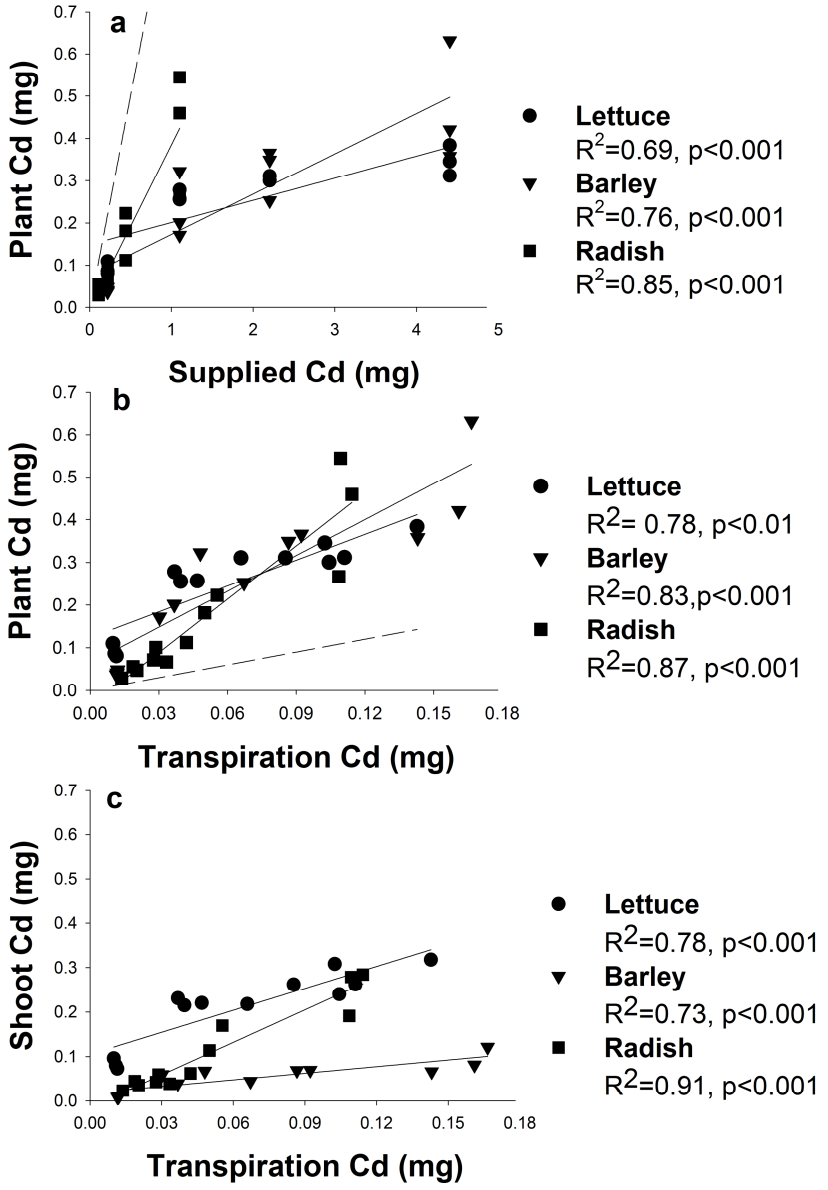

Figure 2.2: Relationships between Cd accumulation and Cd supply in lettuce, barley and radish

(a) The total amount of Cd in each plant is plotted against the total amount of Cd supplied in the growth medium throughout the study period. The dashed line illustrates the maximum amount of Cd that could have been taken up by the plants. (b) The total amount of Cd in each plant is plotted against the amount of Cd in the volume of water that was taken up by each plant. The dashed line represents the maximum Cd available through transpiration. (c) The total amount of Cd in the shoot of each plant is plotted against the amount of Cd in the volume of water that was taken up by each plant. Circles, triangles and squares illustrate lettuce, barley and radish, respectively. The solid lines represent lines of best fit for each plant species.

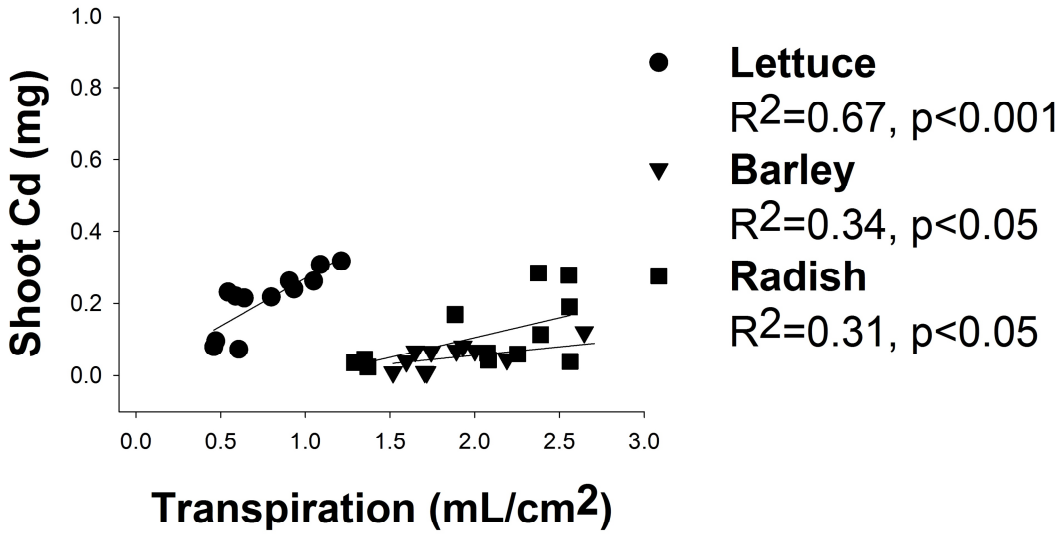

Figure 2.3: Relationship between Cd translocation and transpiration in lettuce, barley and radish

The total amount of Cd in the shoot of each plant is plotted against transpiration per unit leaf area. Circles, triangles and squares illustrate lettuce, barley and radish, respectively. The solid lines represent lines of best fit for each plant species.

strongest correlation (R^2=0.67) was detected in lettuce (Figure 2.3). Although barley and radish transpired three times more water per unit leaf area compared to lettuce, the amounts of Cd in lettuce shoots were comparable to those in radish and up to three times higher than in barley.

2.4 Discussion

The main purpose of this study was to investigate the relationship between Cd content and total volume of water transpired in lettuce, barley and radish. While there was a positive correlation between Cd content and total volume of water transpired in all the three species, the intensity of the relationship was species-specific. I addressed the relationship using three approaches.

First, lettuce, barley and radish transpired different volumes of water throughout the study period and responded differently in terms of Cd accumulation. Radish transpired larger volumes of water compared to lettuce and barley and accumulated the least Cd. Among the three species, barley accumulated the most Cd. When shoot Cd was plotted against the amounts of water transpired per unit leaf area, the strongest correlation was observed in lettuce.

Secondly, budgeting Cd amounts showed that all three species accumulated more Cd than was available through water uptake alone. So, it is confirmed that transpiration alone cannot explain plant Cd accumulation and it is likely that active uptake of Cd^{2+} also took place in the studied species. A number of studies have reported that Cd^{2+} can enter the root through other divalent cation transporters, e.g. Fe^{2+} (Nakanishi et al., 2006) and Ca^{2+} (Zhao et al., 2002). Ueno et al. (2008) studied the uptake and translocation mechanism of Cd^{2+} in *Arabidopsis halleri* and suggested that Cd^{2+} entered the root through an energy-dependent process that is partly shared with Zn^{2+} and/or Fe^{2+} transport. Lombi et al. (2001) investigated the uptake and translocation characteristics of Cd^{2+} and Zn^{2+} for the hyperaccumulator *Thlaspi caerulescens* and raised the possibility of Cd^{2+} transporters in the root cell plasma membranes.

Thirdly, though less total Cd was measured in lettuce compared to barley and radish, lettuce shoots contained higher amounts of Cd than were measured in shoots of the other two species. This pattern could be explained if barley and radish had Cd-restriction mechanisms in the root that minimized translocation to the shoot. One of those mechanisms could be binding Cd^{2+} with phytochelatins, sulphur-rich compounds that are synthesized upon Cd^{2+} exposure in the cytoplasm and vacuole. Salt et al. (1995) reported Cd^{2+}-S complexes in Indian mustard root and noted that most of the Cd^{2+} taken up by Indian mustard was retained in the root. Moreover, different species may accumulate Cd in different compartments within the root. For example, Cd distribution in durum wheat (*Triticum turgidum* var. durum) exposed to 10.0 µM Cd^{2+} was reported to be symplastic (Van der Vliet et al., 2007) whereas Cd distribution in bush beans (*Phaseolus vulgaris*) exposed to 0.5 µM Cd^{2+} was reported as apoplastic (Hardiman and Jacoby, 1984). It is possible that Cd distribution in monocots is mostly symplastic, whereas in dicots Cd is sequestered in the apoplast; however, this idea needs confirmation. If the hypothesis is true then Cd in lettuce may have been translocated to the shoot through apoplastic bypass, whereas in barley Cd was immobilized in the symplast of the root.

Based on the above discussion it is clear that plant Cd accumulation depends on multiple factors, including bulk flow through transpiration, solution Cd concentration and internal compartmentalization of Cd within the root. The relative contribution of each of these factors will determine how much Cd will move into the plant and subsequently be translocated to the aboveground parts. This is consistent with the findings from several other studies conducted on potato, sugar beet, winter wheat (Ingwersen and Streck, 2005) and radish (Kashem and Singh, 2002), where it was shown that, rather than one single factor, Cd accumulation was driven by multiple factors including the ones mentioned above. In our study, regardless of species, plant Cd content increased with increased Cd concentration in the nutrient solution, which is supported by the findings obtained for other species (Ingwersen and Streck, 2005; Salah and Barrington, 2006). The finding that all three plant species showed a positive correlation between shoot Cd and transpiration is in line with the observations from several other studies. Salah and Barrington (2006) studied wheat grown in a range of 0-0.5 mg Cd/L and found that more Cd was taken up by the plants grown under high vapour pressure deficit (VPD) compared to the plants

grown under low VPD and that increased Cd in the soil or nutrient solution increased plant Cd accumulation. Hardiman and Jacoby (1984) exposed 10 day old bush bean (*Phaseolus vulgaris*) in ^{109}Cd for 14 hours either at 68% or 97% relative humidity (RH) and found increased Cd content with increased transpiration. The mean Cd concentrations in the transpirational stream under both RH were similar and the authors suggested that increased Cd transport to the shoot under 68% RH occurred in response to increased mass flow of solutes in the transpirational stream. Ingwerson and Streck (2005) surveyed potato, winter wheat and sugar beet from contaminated sites and found increased Cd concentrations in the years with higher saturation deficit of the atmosphere and they suggested that about 66-82% of the relationship between Cd concentration in the crop and Cd concentration in the soil solution can be explained by the volume of water transpired. On the other hand, Florijn and Beusichem (1993) investigated different inbred lines of maize and found no correlation between Cd content of the shoots and transpiration.

Finally, until a factor unrelated to transpiration is added, Cd translocation from the root to the shoot cannot be explained completely by bulk flow. This factor may be species-specific and includes the ability to either exclude Cd in the rhizosphere through chelation with organic acid exudates from the plant in response to Cd^{2+} exposure or to pass Cd^{2+} through the cell wall into the symplasm using cationic transporters for Ca^{2+}, Zn^{2+}, Cu^{2+} or Fe^{2+} present in the cell membrane (Lombi et al., 2001). Once Cd^{2+} enters the root, it will either enter and bind with the chelators present in the symplast and restrict Cd^{2+} movement to the aboveground part or be translocated directly to the aboveground parts through apoplastic bypass.

2.5 Conclusions

Approximately 85% of the Cd taken up by lettuce accumulated in the leaves, whereas 80% of the Cd in barley was retained in the roots. In radish, Cd was more evenly distributed between aboveground and below ground tissues. Cd accumulation and translocation in lettuce, barley and radish must depend on multiple factors, for example, solution Cd^{2+} concentration, transpiration, loading of Cd into the xylem and/or phloem andinternal compartmentalization of Cd in the root. Preferential retention of Cd^{2+} in the

cell wall or sequestration in the vacuole might explain the observed differences in Cd distribution. So, understanding how and where Cd is stored in the roots is worthy of further investigation as this might enhance our understanding of Cd tolerance and differential translocation in lettuce, barley and radish.

2.5.1 Limitations of the study

It would be valuable to determine whether the observed relationships held true over a broader range of volumes of water that were transpired. A parallel study was done in which plants were grown at 80% RH (data not shown) but the plants did not transpire markedly different volumes of water. Future experiments should include growing plants at lower RH values or under other conditions that would generate higher rates of transpiration.

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Chapter 3

3 Reduced Translocation of Cd from Roots is Associated with Increased Production of Phytochelatins and their Precursors

3.1 Introduction

Phytochelatins (PCs) are enzymatically synthesized peptides in plants that usually consist of three amino acids: glutamic acid (Glu), cysteine (Cys) and glycine (Gly) (Kondo et al., 1984; Grill et al., 1985). The resultant glutathione (GSH) molecule (γ-Glu-Cys-Gly) is transformed into PC by γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase, EC 2.3.2.15), forming the general structural formula of $(\gamma\text{-Glu-Cys})_n$-Gly, where n ranges from 2-11 (Grill et al., 1985; 1987; 1989). The carboxyl-terminal Gly is replaced with serine (Ser) in gramineae hydroxymethyl PCs (Klapheck et al., 1994), β-alanine (β-Ala) in legume homo PCs (Grill et al., 1986), or can either be absent or replaced with Glu in maize (*Zea mays*, Meuwly et al., 1995). PCs are functionally analogous to metallothioneins (MTs), which are produced by animals and some fungi and have been identified in plants ranging from algae to monocots and dicots (Grill et al., 1987).

A number of metal ions are reported to be involved with activation of PC synthase in plants. These include the cations antimony (Sb^{3+}), bismuth (Bi^{3+}), cadmium (Cd^{2+}), copper (Cu^{2+}), gold (Au^{+}), lead (Pb^{2+}), mercury (Hg^{2+}), nickel (Ni^{2+}), silver (Ag^{+}), tin (Sn^{2+}) and zinc (Zn^{2+}) and the anions arsenate (AsO_4^{3-}) and selenite (SeO_3^{2-}) (Grill et al., 1987; 1988). Among these, the strongest activation of the enzyme was observed with Cd^{2+}. The activity of PC synthase is self-regulated in that the product of the reaction (PC) chelates the enzyme-activating metal, thus terminating the enzyme reaction. Once PCs form complexes with metals they will either store the metal in metabolically inactive sites inside the cell (Salt and Rauser, 1995) or release them to apoenzymes, which require these metal ions as cofactors to perform their catalytic activity (Grill et al., 1988). Phytochelatins are thus not only involved in metal detoxification, but also metal homeostasis in plants.

Both PCs and their peptide precursors have a high affinity for metal cations because of the thiol (-SH) groups on the cysteine residues. A number of analytical techniques have been used for the identification and structural analysis of these metal-chelate complexes (Leopold and Gunther, 1997; Scarano and Morelli, 2002; El-Zohri et al., 2005; Chekmeneva et al., 2007; 2008; 2011). In general, the interaction is governed by the binding affinity of thiol groups for metal ions (Checkmeneva et al., 2007; 2008) as well as the availability and complexing capacity of the ligands (Diaz-Cruz et al., 1997; 1998; Cruz et al., 2002; Kobayashi and Yoshimura, 2006; Checkmeneva et al., 2007; 2008).

The binding stoichiometry of the metal-PC_n complexes has also been studied (Diaz-Cruz et al., 1997; 1998; Kobayashi and Yoshimura, 2006; Chen et al., 2007; Chekmeneva et al., 2007; 2008; 2011). It was found that an increase in the number of thiol groups in a molecule produces an increase in the binding capacity, i.e. the number of metal ions that can be bound to a PC_n molecule (Chekmeneva et al., 2011). Chen et al. (2007) studied Cd^{2+}-PC_n complexes from a Cd hyperaccumulator, *Brassica chinensis,* and reported the binding stoichiometries as 1:1 to 3:1 based on the availability of Cd^{2+} and thiol groups in the Cd^{2+}-PC_n complexes in the cytosol.

The ability of metal ion-PC_n complexes to sequester metals in metabolically inactive sites depends on the stability of the complex. Chekmeneva et al. (2007; 2008; 2011) measured stability constant values of Cd^{2+}-PC_n complexes using different techniques and concluded that the stability increases with higher chain lengths, up to PC_3. Beyond PC_3, the stability of the complexes stays the same due to the fact that four or more thiol groups can saturate the coordination number of Cd^{2+}, which is usually tetrahedral.

Previous studies have reported Cd^{2+}-induced PC synthesis (Grill et al., 1985; Rainieri et al., 2005; Wang and Wang, 2011) and identified Cd^{2+}-PC_n complexes either under laboratory conditions (Kobayashi and Yoshimura, 2006; Chekmeneva et al., 2007; 2008; 2011) or from plants in their native environment (Scarano and Morelli, 2002; Chen et al., 2007) as evidence for the proposed mechanism (Sanità di Toppi and Gabbrielli, 1999) involved in Cd detoxification in plants.

In this study the total amount of thiol-containing PCs and their precursors produced in the roots and shoots of lettuce and barley was measured and the binding stoichiometries of possible Cd^{2+}-PC_n complexes were used to estimate the theoretical efficiency of thiol-containing molecules in binding Cd^{2+} to understand the role of PCs and their precursors against differential Cd accumulation in barley and lettuce. The plant species were chosen because, in a previous experiment, lettuce and barley seedlings showed consistent differences in the proportions of the total Cd taken up in the plant that were translocated to the shoot. When grown in hydroponic nutrient solution containing 0.10 to 2.0 µM Cd, the proportions of Cd translocated to the shoots ranged from 19.0±0.2% to 25.2±4.9 % in barley and from 78.1±4.2% to 90.0±1.4% in lettuce (Chapter two; Akhter and Macfie, 2012). The mechanisms that control Cd translocation have not yet been determined. In lettuce and barley (Chapter two; Akhter and Macfie, 2012), rice (*Oryza sativa*, Uraguchi et al., 2009) and maize (Florijn and Beusichem, 1993) increased translocation of Cd to the shoots in some plants could not be explained by greater volumes of water transpired. Uraguchi et al. (2009) measured higher concentrations of Cd in the xylem of rice with increased translocation of Cd but neither those plants nor varieties of durum wheat (*Triticum turgidum* var durum, Adeniji et al., 2010) with higher concentrations of Cd in the shoots took up more Cd from the growth medium than did the varieties with less Cd in the shoots. Increased translocation of Cd from the roots appears to be related to increased xylem loading and/or increased retention of Cd in the roots. Complexation of Cd^{2+} with PC_n or their precursor complexes (Cys, Glu, and γ-Glu-Cys) in roots could contribute to reduced xylem loading and reduced translocation. Thus, the hypothesis that increased accumulation of Cd in the roots of barley is related to increased concentrations of phytochelatin (PC_{2-4}) and its precursor peptides was tested in this study.

3.2 Methods and Materials

3.2.1 Chemicals

Chemicals, stock solutions and reagents used were of analytical grade. Diethylenetriamine-pentaacetic acid (DTPA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), N-acetyl-L-cysteine (NAC), glutathione (GSH), γ-glutamylcysteine (γ-Glu-Cys or γ-EC), L-cysteine (Cys), Tris(2-carboxyethyl)phosphine

hydrochloride (TCEP), monobromobimane (MBrB), methanesulfonic acid (MSA), and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Oakville, ON, Canada); hydrochloric acid (HCl) and acetonitrile (ACN) were obtained from Caledon (Georgetown, ON, Canada). Phytochelatin standards for PC_2, PC_3 and PC_4, each with $\geq$ 95% purity, were obtained from AnaSpec (Fremont, CA, USA), who used solid phase peptide synthesis to generate the PCs. PC_{2-4} were chosen for analysis because they form the primary Cd^{2+}-PC_n complexes in plants (Scarano and Morelli, 2002; Chen et al., 2007; Sadi et al., 2008). All solvents and ACN were filtered with a 0.45 µm filter (Type HA, Millipore Corporation, Etobicoke, ON, Canada). Water was purified by a Milli-Q system. All glassware was washed in soapy tap water, rinsed in tap water, soaked in 10% (v/v) hydrochloric acid overnight, rinsed in RO (reverse osmosis) water and air-dried before use.

3.2.2 Germination and growth conditions

Leaf lettuce (*Lactuca sativa* L. cv. Grand Rapids) and barley (*Hordeum vulgare* L. cv. CDC McGwire, hulless 2-row feed barley) seeds were placed on moist (RO water) filter paper in Petri dishes and placed in the dark at room temperature. When the radicles were approximately 1.0 cm long (24 - 36 h), seedlings were transferred to sand-filled pots and watered with nutrient solution adjusted to pH 6.0. The nutrient solution contained 1.0 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1.0 mM K_2HPO_4, 0.40 mM KNO_3, 0.30 mM $Mg(NO_3)_2 \cdot 6H_2O$, 0.30 mM NH_4NO_3, 0.10 mM K_2SO_4, 10.0 µM $FeCl_3 \cdot 6H_2O$, 10.0 µM Na_2EDTA, 6.0 µM H_3BO_3, 2.0 µM $MnCl_2 \cdot 4H_2O$, 0.50 µM $ZnSO_4 \cdot 7H_2O$, 0.15 µM $CuSO_4 \cdot 5H_2O$ and 0.10 µM Na_2MoO_4.

Potted seedlings were kept in a growth chamber set to 21ºC, 60% relative humidity, and a 16 h day length. The light intensity was 187±1.5 µmol/m^2/s. The seedlings were transferred to 1.4 L glass jars after 1 week in sand culture. Two seedlings were secured in the lid of a jar with a 0.5 x 1 x 6 cm piece of foam, and each jar was covered with black cloth to prevent algal growth. The jars were filled with nutrient solution to which either no Cd (n=3) or 1.0 µM $CdCl_2.5H_2O$ (hereafter referred to as $CdCl_2$, n=6) was added, and the pH was adjusted to 6.0 using concentrated HCl. Each jar was connected to an aeration

system and the plants were provided with fresh nutrient solution (including the corresponding Cd treatment) every second day. On the 28th day in hydroponic culture, seedlings from three of the 1.0 μM $CdCl_2$ treatments were moved into new jars of aerated nutrient solution with 5.0 mM $CdCl_2$ (pH 6.0) for 1 h. At harvest, the roots were separated from the shoots from one plant in each jar, rinsed in RO water and oven dried (60ºC) to constant weight and stored for Cd analysis. The Cd measured in these roots represented the total amount accumulated. The Cd in the apoplast of the roots from the other plant was desorbed using $CaCl_2$ (Buckley et al., 2010, with some modifications). Specifically, the roots were rinsed in RO water and transferred to 900 mL of 5.0 mM $CaCl_2$ at 0°C (ice water bath) for 30 min. After 30 min of desorption, the roots were separated from the shoots, rinsed in RO water and oven dried (60ºC) to constant weight and stored for Cd analysis. The amount of Cd in these tissues represented the amount in the symplast. Control seedlings were treated with the same procedure except that RO water was used instead of $CaCl_2$. The amount of Cd in the apoplast was calculated as apoplastic Cd = total Cd - symplastic Cd. As a control check, the concentration of Cd in the $CaCl_2$ wash was also measured.

In another experiment, a separate batch of seedlings was grown following the same procedures mentioned above except that individual seedlings were transferred to glass jars. At harvest, fresh weights of roots and shoots were recorded and a 1.0 g subsample of each tissue type was flash frozen in liquid nitrogen and stored at -80ºC for PC analysis. The remainder of the root and shoot samples were oven dried (60ºC) to constant weight and stored for Cd analysis.

3.2.3 Extraction of thiol-containing molecules

Thiol-containing compounds were extracted following the method of Sneller et al. (2000) with some modifications. Frozen (-80ºC) root and shoot samples were ground in liquid nitrogen (N_2) using a mortar and pestle, and 0.10 g of each sample was immediately placed in an individual microcentrifuge tube containing 1.5 mL of 6.3 mM DTPA with 0.1%, v/v, TFA and 25 μL of 20 mM TCEP (4ºC). The mixture was sonicated in ice water (Cole-Parmer ultrasonic system, model no. 8893-21, Montreal, QC, Canada) for 25 min and the supernatant was collected after centrifugation at 15000×g for 60 min at 4ºC.

The thiol groups were derivatized (section 3.2.5) immediately and analysed using HPLC (section 3.2.6). The unused portion of each sample was returned to the -80ºC freezer.

3.2.4 Preparation of thiol-containing standards

Standards and reactant solutions were prepared according to the procedure described in Minocha et al. (2008) with some modifications. Stock solutions of 1.0 mM of each thiol-containing standard (Cys, Glu, γ-Glu-Cys, PC_2, PC_3, PC_4 and NAC [N-acetyl-cysteine], an internal standard, were prepared using deionised water (RO water) and stored in the dark at -20°C. NAC was necessary because duplicate measurements of each standard had peak areas that varied by up to 3%. Adjusting the NAC value for each standard and experimental sample to a pre-determined value ensured that this instrument variability did not affect quantification of thiol-containing molecules. The pre-determined value was equal to the average NAC peak area obtained for three independent NAC samples.

The concentrations used to prepare standard curves and establish detection limits ranged from 0 to 200 µM for Cys, γ-Glu-Cys, GSH, PC_2, and PC_3 and 0 to 100 µM for PC_4. At concentrations higher than 100 µM, the chromatographic peaks for PC_4 were off-scale. To make the series of standards, the stock solutions were diluted with 6.3 mM DTPA with 0.1%, v/v, TFA (extraction buffer). Thiol-containing standards were prepared fresh on the day of use, derivatized immediately (section3.2.5) and analysed using HPLC (section 3.2.6). Thiol-containing molecules were quantified using five-point calibration curves (Table 3.1). The slope for PC_2 was lower than expected. Repeated preparation of this component resulted in consistently low slope values, which indicates that the molecule may have degraded (oxidized). If this was the case, then the calculated concentrations of PC_2 in the experimental samples might be slightly higher than the actual values; however, the relative amounts of PC_2 among our experimental treatments would be unaffected. A standard mixture containing monothiols (Cys, γ-EC and GSH), an internal standard (NAC) and polythiols (PC_2, PC_3 and PC_4) was also run.

3.2.5 Derivatization of thiol groups

The thiol-containing compounds were derivatizated with MBrB following the procedures of Rijstenbil and Wijnholds (1996) and Sneller et al. (2000), as described in Minocha et

al. (2008). HEPPS buffer (200 mM) was prepared in 6.3 mM DTPA set to pH 8.2. Then, 615 µL of this solution was mixed with 25 µL of 20 mM TCEP solution, which was prepared fresh each day of use in 1.0 M HEPPS buffer and used as a reducing agent in the reaction mixture. To this mixture, samples or standards (250 µL) as well as NAC (10 µL of 0.50 mM) were added and the mixture was pre-incubated at 45ºC for 10 min. This procedure converted the disulfide bonds (S-S) to sulfhydryl (-SH) bonds so that the thiol groups were in a reduced state before MBrB derivatization. MBrB is light-sensitive and hence the stock solution was covered with aluminum foil and kept in the dark at 4ºC until use. MBrB was added (10 µL of 50 mM prepared in ACN) to the mixture and the tube was placed the dark at 45ºC for 30 min. The reaction was terminated by adding 100 µL of 1M MSA; the solution was filtered (0.2 µm) before HPLC analysis.

3.2.6 HPLC instrumentation and chromatographic condition

The HPLC instrument used was an Agilent Technologies 1200 series system with the following components: G1311A quaternary pump, G1322A degasser, G1367B auto sampler, G1330B FC/ALS Therm, G1315D diode array detector (DAD), G1321B fluorescence detector (FLD), and Chemstation software. The column used was a C_{30}, YMC-Carotenid ^{TM} column with 3 µm particle size (4.6×250 mm, Waters). The injection volume was 50 µL. The excitation and emission wavelengths were set at 390 and 490 nm, respectively. Thiol-containing molecules were separated by using two solvents: (A) 0.1% TFA in RO water and (B) ACN. The details of the gradient profile are given in Table 3.2. Total run time for each sample was 60 min including column cleaning. The flow rate was set at 1 mL/min throughout the run time. The detection limit (3 × average noise level) was calculated from the lowest concentration of each standard visible in the chromatogram (Table 3.1). Finally, data were integrated using Chemstation software.

Table 3.1: Linear ranges, r^2 and slope values for standard curves of the thiol compounds

Component name	Detection limit (nmol/50 µL)	Linear range (nmol/50 µL)	Coefficient of determination, r^2	slope
Cys	0.02	0 - 1.67	0.99	44.39
GSH	0.02	0 - 1.67	0.99	26.67
γ-EC	0.02	0 - 1.67	0.99	25.68
PC_2	0.10	0 - 1.67	0.99	4.88
PC_3	0.01	0 - 1.67	0.98	79.63
PC_4	0.01	0 - 0.83	0.94	73.32

Table 3.2: Solvent gradient profile used in the separation of MBrB-derivatized thiols using HPLC

Time (min)	Solvent A (by volume) (0.1% TFA)	Solvent B (by volume) (ACN)
0.1	95.0	5.0
40.0	70.0	30.0
41.0	40.0	60.0
45.0	0	100.0
55.0	0	100.0
56.0	95.0	5.0
60.0	95.0	5.0

3.2.7 Estimation of Cd^{2+}-thiol-complexation

The capacity for thiol-containing molecules to bind Cd^{2+} ions in the samples was estimated. This was done based on the measured amounts of Cd^{2+}, PCs and PC-precursors as well as the expected ratios of Cd^{2+} and PCs in the potential Cd^{2+}-PC_n complexes. The ratios used were 1:1 for Cd^{2+}-Cys, Cd^{2+}-(γ-Glu-Cys) and Cd^{2+}-GSH, 2:1 for Cd^{2+}-PC_2 and 3:1 for Cd^{2+}-PC_{3-4} (Chen et al., 2007; Chekmeneva et al., 2011).

3.2.8 Cadmium content

The concentrations of Cd in samples were determined following the method in section 2.2.4.

3.2.9 Statistical analyses

One-way ANOVA followed by Tukey tests were used to detect significant (*$P<0.05$)* effects of Cd treatment on Cd content and thiol compound content in the shoot and root tissues and for differences between apoplast and symplast Cd content. The coefficient of determination (R^2) was calculated for each thiol standard curve and used to assess the precision of each standard curve. The graphics and statistical analyses were done in SigmaPlot (version 11.0).

3.3 Results

3.3.1 Cadmium content

Plants grown in control solution did not contain measureable amounts of Cd, except for roots of barley in which Cd was just above the detection limit (Table 3.3). Low concentrations (0.09 to 0.33 mg/g) and amounts (0.07 to 0.45 mg) of Cd were measured in shoots and roots of barley and lettuce from the 1.0 µM $CdCl_2$ treatment, with roots having 1.5- to 2-fold higher concentrations than shoots. When plants were exposed to 5.0 mM $CdCl_2$ for 1 h prior to harvest, concentrations of Cd in shoots increased by 50% in barley and 25% in lettuce while concentrations of Cd in roots increased 5-fold in barley and 50-fold in lettuce, compared to plants from the 1.0 µM $CdCl_2$ treatments. The patterns were similar for the total amount of Cd (amount = Cd concentration × biomass); amounts of Cd increased in response to the 5.0 mM $CdCl_2$ treatment and roots contained

Table 3.3: Concentration and amount of Cd in barley and lettuce grown in different Cd treatments.

	Cd Treatment	Barley				Lettuce			
		Shoot		Root		Shoot		Root	
Cd concentration (mg/g)	No Cd	<dl [a]		0.004 (0.001) [a]		<dl [a]		<dl [a]	
	1 µM	0.085 (0.010) [b]		1.177 (0.010) [b]		0.206 (0.011) [b]		0.326 (0.026) [a]	
	5 mM	0.133 (0.002) [c]		6.339 (0.459) [c]		0.268 (0.007) [c]		15.46 (0.599) [b]	
	One-way ANOVA	p	$F_{(2,8)}$	p	$F_{(2,8)}$	p	$F_{(2,8)}$	p	$F_{(2,8)}$
		0.001	130.86	0.001	33.51	0.001	295.26	0.001	670.71
Total Cd (mg)	No Cd	<dl [a]		0.003 (0.001) [a]		<dl [a]		<dl [a]	
	1 µM	0.142 (0.012) [b]		0.448 (0.036) [a]		0.223 (0.037) [b]		0.065 (0.012) [a]	
	5 mM	0.243 (0.011) [c]		2.325 (0.368) [b]		0.259 (0.013) [b]		3.032 (0.319) [b]	
	One-way ANOVA	p	$F_{(2,8)}$	p	$F_{(2,8)}$	p	$F_{(2,8)}$	p	$F_{(2,8)}$
		0.001	168.70	0.001	33.51	0.001	34.74	0.001	93.43
% of total Cd retained in the root	No Cd	<dl				<dl			
	1 µM	76 (1.7)				23 (1.2)			
	5 mM	90 (0.9)				92 (0.4)			

Plants were grown with 0 or 1.0 µM $CdCl_2$ for 28 d. Half of the plants grown with Cd were transferred to 5.0 mM $CdCl_2$ for 1 h immediately prior to harvest. Within each tissue, different lower case letters indicate significance differences in Cd concentration and Cd accumulation, as determined by post-hoc Tukey tests. Values are mean (SE), n=3 for each treatment, dl = detection limit.

higher concentrations of Cd than did shoots.

The two species differed in their relative translocation of Cd to shoots. In the 1.0 μM $CdCl_2$ treatment (Table 3.3), barley stored 76% of total Cd in the root and translocated only 24% to the shoot; in contrast, lettuce stored only 23% of the total Cd in the root and translocated the rest to the shoot. Regardless of the species, plants stored ~ 90% of the total Cd in the root when exposed to 5.0 mM $CdCl_2$ for 1 h; however, at the end of this treatment barley plants appeared healthy and stood straight whereas lettuce plants lost vigour and wilted.

3.3.2 Apoplastic and symplastic Cd

After desorption of Cd from the apoplast, the concentrations of Cd remaining in plants grown with a chronic, low concentration of Cd were below the detection limit of the ICP-AES (data not shown), thus plants given the acute exposure to 5.0 mM $CdCl_2$ were used to estimate the distribution of Cd within the roots. Because proportionally more Cd might be expected to be in the apoplast of plants given an acute exposure to a very high concentration of Cd, the amounts of symplastic Cd for the plants from the 1.0 μM $CdCl_2$ treatment in Table 3.5 are likely to be slight underestimates. Concentrations of Cd were higher in lettuce roots compared to barley roots (Figure 3.1a). In lettuce, the Cd concentration was 2-fold higher in the apoplast compared to symplast, whereas no difference was detected in barley (Figure 3.1a). When the total amount of Cd in each root compartment was calculated, there were no differences in total Cd accumulation between the species (Figure 3.1b), each accumulated about 0.5 mg Cd. In barley, Cd was evenly distributed between the apoplast and symplast whereas lettuce stored only 35% of the total root Cd in the symplast and the rest was bound within the apoplast (Figure 3.1b).

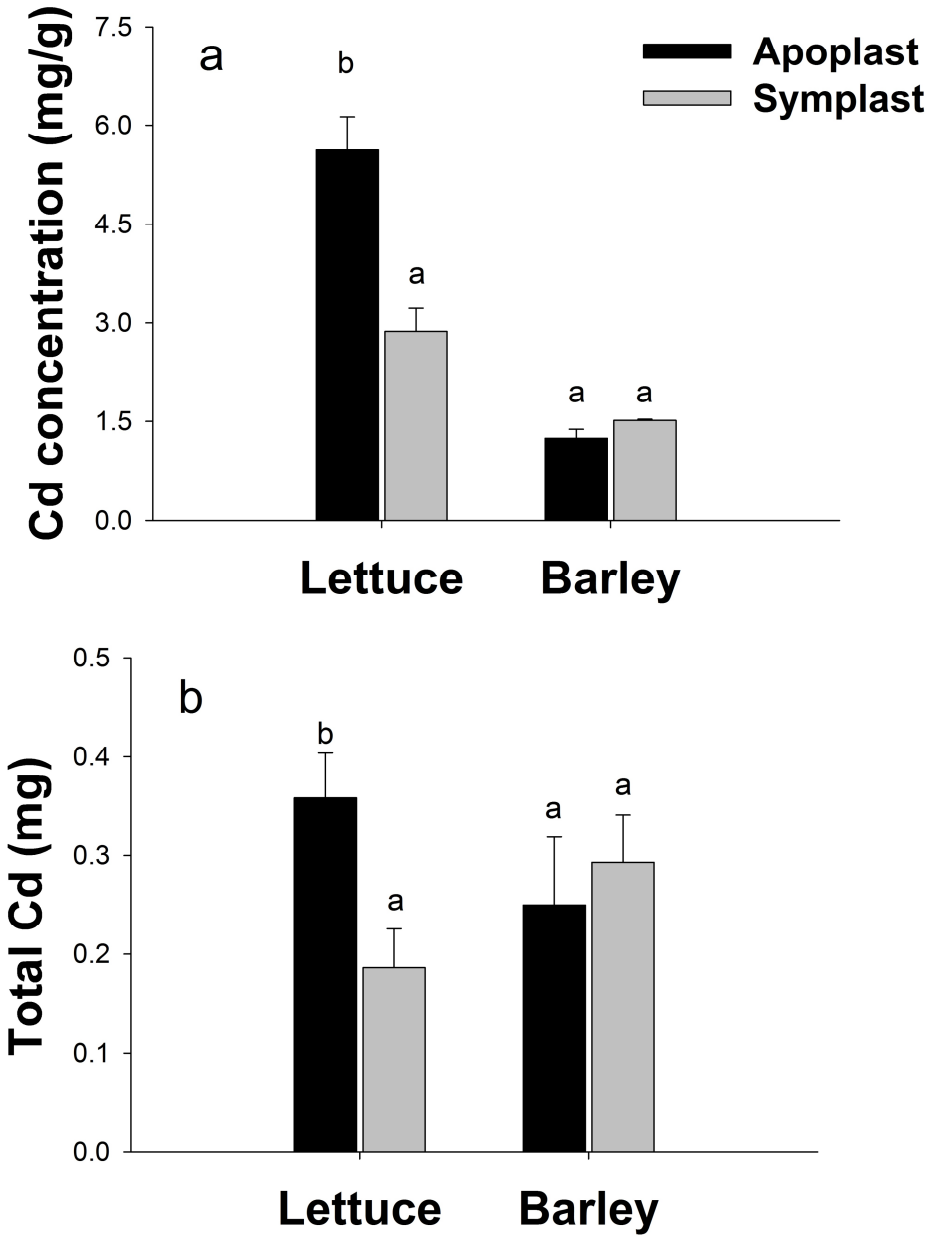

Figure 3.1: (a) Concentration (+SE, mg/g) and (b) total amount (+SE, mg) of Cd in the apoplast and symplast compartments in lettuce and barley root

The plants were grown in 1.0 µM $CdCl_2$ for 28 d before exposed to 5.0 mM $CdCl_2$ for 1 h at harvest. Within each species, different lower case letters indicate significance differences in Cd accumulation, as determined by one-way ANOVA and post-hoc Tukey tests ($p < 0.05$).

3.3.3 HPLC profile of thiol containing compounds

The C_{30} column used in this study improved the resolution of peaks compared to other MBrB-based derivatization methods that used a C_{18} column (e.g., Minocha et al., 2008; Thangavel et al., 2007). Identification of the components was confirmed by spiking the reaction blank and standard mixture with individual components, one at a time. A very broad reagent peak was observed in the chromatograms at approximately 28 min. This peak was also observed in other MBrB-based derivatization studies (e.g., Thangavel et al., 2007; Minocha et al., 2008). Kawakami et al. (2006) identified this peak as tetramethylbimane (Me_4B) and reported that this compound was used during the synthesis of MBrB.

3.3.4 Monothiols and PCs in plant tissues

The total amount of each monothiol and PC in the shoot and root tissues was calculated by multiplying the concentration of each thiol-containing compound by the corresponding tissue mass (Table 3.4, with trends summarized in Figure 3.2). In barley shoots, the amounts of Cys, GSH and PC_4 were lowest in plants exposed to 1.0 µM $CdCl_2$ for 4 weeks. When the same plants were exposed to 5.0 mM $CdCl_2$ for 1 h prior to harvest, the amounts of Cys, GSH and PC_4 returned to control values. For all treatments, the total amount of PC_4 in the shoots was very low. Cadmium treatment did not affect the amounts of γ-EC in the shoots. Each of PC_2 and PC_3 were below the detection limit in barley shoots.

In barley roots grown in control solution, all monothiols (except GSH) and PCs were below the detection limit (Table 3.4, with trends summarized in Figure 3.2). The amounts of GSH in barley did not vary with Cd treatment but each of Cys, γ-EC, PC_2, PC_3 and PC_4 increased in response to Cd. The amounts were the same for plants in the 1.0 µM $CdCl_2$ treatment and the 5.0 mM $CdCl_2$ treatment, except for Cys. Plants synthesized three to four times more Cys when exposed to 5.0 mM $CdCl_2$ for 1 h prior to harvest.

Table 3.4: Molar amounts of phytochelatins (PC_2, PC_3, and PC_4) and their precursor (Cys, γ-EC, and GSH) monothiols in the shoot and root tissue extracts of barley and lettuce exposed to different Cd treatments

Tissue	Cd Treatment	Cys (µmol)	γ-EC (µmol)	GSH (µmol)	PC_2 (µmol)	PC_3 (µmol)	PC_4 (µmol)
Barley shoot	No Cd	5.74 (0.80) [b]	0.99 (0.24) [a]	20.10 (3.29) [b]	<dl	<dl	0.27 (0.03) [b]
	1 µM Cd	1.79 (0.12) [a]	0.51 (0.03) [a]	8.00 (0.08) [a]	<dl	<dl	0.13 (0.02) [a]
	5 mM Cd	5.93 (0.11) [b]	0.79 (0.19) [a]	14.75 (0.23) [ab]	<dl	<dl	0.26 (0.01) [b]
	One-way ANOVA	p 0.001, $F_{(2,8)}$ 24.59	p 0.24, $F_{(2,8)}$ 1.84	p 0.01, $F_{(2,8)}$ 10.13			p 0.007, $F_{(2,8)}$ 13.07
Barley root	No Cd	<dl [a]	<dl [a]	2.83 (0.42) [a]	<dl [a]	<dl [a]	<dl [a]
	1 µM Cd	0.20 (0.04) [b]	0.72 (0.09) [b]	2.09 (0.19) [a]	1.60 (0.15) [b]	0.05 (0.01) [b]	0.11 (0.01) [b]
	5 mM Cd	0.91 (0.09) [c]	0.74 (0.08) [b]	2.70 (0.15) [a]	2.31 (0.24) [c]	0.05 (0.003) [b]	0.12 (0.01) [b]
	One-way ANOVA	p 0.001, $F_{(2,8)}$ 70.73	p 0.001, $F_{(2,8)}$ 36.77	p 0.22, $F_{(2,8)}$ 1.99	p 0.001, $F_{(2,8)}$ 52.44	p 0.002, $F_{(2,8)}$ 22.93	p 0.001, $F_{(2,8)}$ 66.49
Lettuce shoot	No Cd	8.60 (0.87) [b]	<dl	8.35 (1.14) [b]	<dl	<dl	0.18 (0.01) [b]
	1 µM Cd	3.05 (0.50) [a]	<dl	3.95 (0.54) [a]	<dl	<dl	0.09 (0.02) [a]
	5 mM Cd	3.89 (0.01) [a]	<dl	3.34 (0.05) [a]	<dl	<dl	0.30 (0.004) [c]
	One-way ANOVA	p 0.01, $F_{(2,8)}$ 26.66		p 0.005, $F_{(2,8)}$ 14.07			p 0.001, $F_{(2,8)}$ 64.54
Lettuce root	No Cd	0.13 (0.01) [a]	<dl	0.44 (0.06) [b]	<dl	<dl	<dl
	1 µM Cd	0.26 (0.01) [a]	<dl	0.21 (0.03) [a]	<dl	<dl	<dl
	5 mM Cd	0.09 (0.004) [a]	<dl	0.39 (0.001) [b]	<dl	<dl	<dl
	One-way ANOVA	p 0.059, $F_{(2,8)}$ 4.73		p 0.01, $F_{(2,8)}$ 9.75			

Plants were grown with 0 or 1.0 µM $CdCl_2$ for 28 d. Half of the plants grown with Cd were transferred to 5.0 mM $CdCl_2$ for 1 h immediately prior to harvest. Within each thiol, different lower case letters indicate significance differences in thiol amounts, as determined by post-hoc Tukey tests. Values are mean (SE), n=3 for each treatment, dl = detection limit.

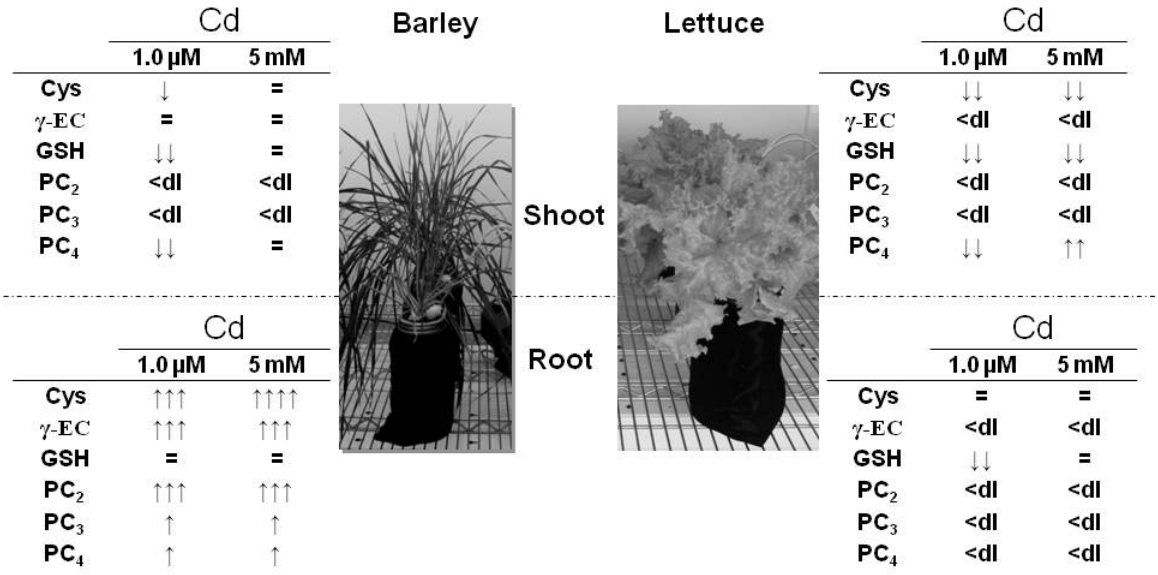

Figure 3.2: Schematic presentation of the relative changes in molar amounts of phytochelatins (PC_2, PC_3, and PC_4) and their precursor monothiols (Cys, γ-EC, and GSH) in plants from the two Cd treatments relative to the corresponding control plants, as reported in Table 3.4

Within each species, = indicates no change relative to control, upward and downward arrows indicate increases and decreases relative to control, respectively. = indicates no change, one arrow indicates a change in the order of 30-45%, two arrows indicate a change in the order of 50%, three arrows indicate a change of about 100% and four arrows indicate a change of about 500%, <dl indicates below detection limit.

Lettuce shoots contained high amounts of Cys and GSH when grown in control solution and, as in barley, the amounts were lower in plants from the 1.0 µM $CdCl_2$ treatment (Table 3.4, with trends summarized in Figure 3.2). However, unlike in barley, the amounts of Cys and GSH did not return to control values after the acute 5.0 mM $CdCl_2$ treatment. Similar to barley, the amounts of γ-EC, PC_2 and PC_3 were below detection limit and a very low amount of PC_4 was measured in lettuce shoots.

Only Cys and GSH were detected in roots of lettuce (Table 3.4, with trends summarized in Figure 2). There were no effects of Cd treatment on Cys production. GSH was reduced in plants in the 1.0 µM $CdCl_2$ treatment compared to the control plants and returned to the control value after the 5.0 mM $CdCl_2$ treatment (Table 3.4).

The total amount of thiol-containing compounds in control plants was higher in barley (29.9±4.8 µmol) than in lettuce (17.7±2.1 µmol); barley contained higher amounts of γ-EC and GSH but lower amounts of Cys relative to lettuce (Table 3.4). PCs were below detection limit in lettuce root, whereas barley root synthesized PCs upon Cd exposure. It thus appears that barley was more efficient in synthesizing thiol-containing molecules compared to lettuce.

3.3.5 Estimating the formation of Cd^{2+}-thiol complexes

Phytochelatins are synthesized in the root symplast and can bind Cd^{2+} in this compartment only. Based on the results of the apoplast-symplast study (Figure 3.1), the total number of moles of Cd in the root and the moles of Cd theoretically present in the symplast of barley and lettuce root were calculated (Table 3.5). The maximum number of moles of Cd^{2+} that could theoretically be chelated by the PCs as well as the monothiols that were measured in the barley and lettuce roots was calculated (using data from Table 3.4). The ability of thiol groups to bind Cd^{2+} was calculated based on published information on the binding stoichiometry of Cd^{2+}- PC_n complexes (Cruz et al., 2002; Chekmeneva et al. 2007; 2008; 2011). It was assumed that all of the Cd estimated to be in the symplast was available to interact with all of the thiol groups and that no other types of molecule formed a complex with Cd. While this is no doubt an overestimate of the actual amount of Cd^{2+}-available for complexation, it provides an estimate of the

Table 3.5: Estimated amounts of Cd^{2+} that could be complexed with the thiol-containing molecules in the symplast of roots of barley and lettuce

Species	Cd Treatment	Total root Cd (µmol)	Symplast Cd (µmol)	Cd^{2+} chelated by PCs (µmol)	% Cd^{2+} chelated by PCs	Total Cd^{2+} chelated by monothiols and PCs (µmol)	% Cd^{2+} chelated by monothiols and PCs
Barley	No Cd	0.03 (0.01)	0.01 (0.01)	0	0	2.83 (0.42)	100
	1.0 µM	3.99 (0.32)	2.15 (0.17)	3.66 (0.34)	100	6.68 (0.63)	100
	5.0 mM	20.69 (3.27)	11.17 (1.77)	5.12 (0.53)	45.8	9.48 (0.83)	84.9
Lettuce	No Cd	<dl	<dl	0	0	0.57 (0.06)	100
	1.0 µM	0.58 (0.11)	0.20 (0.03)	0	0	0.46 (0.10)	100
	5.0 mM	26.97 (2.84)	9.30 (0.98)	0	0	0.49 (0.004)	5.3

Total Cd includes both apoplastic and symplastic Cd. Symplast Cd was estimated using data from Figure 3.1. The amounts of Cd^{2+} in the symplast that could form complexes with phytochelatins (PC_{2-4}) and monothiols (Cys, γ-EC and GSH) were calculated assuming that all of the Cd in the symplast was in the Cd^{2+} form and all thiol groups were available to interact with all Cd^{2+} ions. The thiol/Cd^{2+} stoichiometries used were 1:1, 1:2 and 1:3 for the monothiols-Cd^{2+}, PC_2-Cd^{2+}, and PC_{3-4}-Cd^{2+} complexes, respectively. Barley and lettuce were grown with 0 or 1.0 µM $CdCl_2$ for 28 d. Half of the plants grown with Cd were transferred to 5.0 mM $CdCl_2$ for 1 h immediately prior to harvest. Molar amounts are mean (SE), n=3 for each treatment, dl=detection limit.

maximum potential for Cd^{2+} to form complexes with PCs and their precursors. I determined that PCs had the potential to chelate as much as 100% of the symplastic Cd^{2+} in barley roots exposed to 1.0 µM $CdCl_2$ for 28 days (Table 3.5). When the same plants were exposed to 5.0 mM $CdCl_2$ for 1 h prior to harvest, thiol-containing compounds could form complexes with only 46% of the total amount of symplastic Cd^{2+}. When monothiols were included as potential Cd^{2+} chelators, 100% (1.0 µM $CdCl_2$) and 85% (5.0 mM $CdCl_2$) of the symplastic Cd^{2+} could have been chelated with thiol-containing molecules. In the case of lettuce, no PCs were detected under experimental conditions and only monothiols were present (Table 3.5). At 1.0 µM $CdCl_2$, these monothiols could theoretically form complexes with 100% of the total symplast Cd^{2+} in the lettuce root. For the lettuce exposed to 5.0 mM $CdCl_2$ for 1 h, synthesis of monothiols was unchanged and the efficiency of complexation with Cd^{2+} dropped to 5%.

3.4 Discussion

The potential role of PC_{2-4} and their precursor peptides in differential Cd accumulation in lettuce and barley was tested in the present study by growing plants under two conditions: chronic (28 d) exposure to a low, environmentally relevant concentration (1.0 µM) of Cd and acute (1 h) exposure to a high concentration (5.0 mM) of Cd. Chronic exposure was used to evaluate the 'steady state' status of the various peptides under mild Cd stress; acute exposure was used to evaluate the initial response to potential Cd toxicity.

3.4.1 Differential Cd accumulation

The distribution of Cd differs between lettuce and barley. When grown with a chronic, low concentration of Cd only 24% of the total Cd taken up by lettuce was retained in the root, whereas 76% of the total Cd in barley was retained in the root (Table 3.3). This confirms the previous report of differential translocation of Cd in these two species (Chapter two; Akhter and Macfie, 2012). When plants were exposed to a very high concentration of Cd for 1 h, over 90% of the total Cd was found in the root for both species, likely reflecting lack of time for the Cd to be translocated to the shoot.

The species also differed in the localization of Cd within the root. Approximately two thirds of the total Cd taken up by lettuce roots from the acute Cd treatment was predicted to be in the loosely bound (apoplast) fraction. In contrast, Cd in barley roots appeared to be evenly distributed between the apoplast and symplast. Thus, these species provide a good system in which to examine the role of metal-binding molecules in differential translocation of Cd.

3.4.2 Phytochelatins

The synthesis of PCs in response to Cd has been reported in a number of studies conducted on various species including a marine diatom (*Thalassiosira nordenskioeldii*, Wang and Wang, 2011), freshwater green alga (*Scenedesmus vacuolatus*, Le Faucheuret al., 2005), tobacco cell culture (*Nicotiana tabacum*, Zitka et al., 2011), bread wheat (*Triticum aestivum*, Rainieri et al., 2005), rice (Nocito et al., 2011) and broad bean (*Vicia faba*, Čabala et al., 2011). The fact that no PCs were detected in the roots of lettuce grown in either Cd treatment (Table 4) indicates that PCs were not involved in Cd^{2+}chelation and accumulation in lettuce root. In contrast to the results of this study, Maier et al. (2003) reported PCs in concentrations of ~0.10 μmol g^{-1} fresh weight in roots of romaine lettuce (*L. sativa* var longifolia) upon exposure to 25 nM $CdCl_2$. It is possible that PCs in this study degraded during sample preparation; the PCs were extracted from frozen tissue (liquid nitrogen followed by storage at -80°C) rather than immediately harvested tissue, and Maier et al. (2003) showed that up to 50% of the PCs can be lost during freezing.

Most of the Cd taken up by lettuce was translocated to the shoot. However, low amounts ($\leq$ 0.3 μmol) of only one PC, PC_4, were detected in lettuce shoots making it unlikely that PCs were a major contributor to Cd^{2+} detoxification in the shoot either. Maier et al. (2003) also reported low concentrations of total PCs in romaine lettuce shoots (~0.02 to 0.25 μmol/g fresh weight) exposed to Cd. The PCs in control plants may have been produced in response to the $Zn^{2+}$and Cu^{2+} in the nutrient solution. Along with Cd^{2+}, these metal ions can also induce the synthesis of PCs (Grill et al., 1987). The amounts of PCs synthesized in response to nutrient cations are expected to be low but it was surprising

that only PC_4 was detected in plants from control and $CdCl_2$ treatments. It was expected that PC_2 was also present in lettuce leaves but was below detection limit. Under experimental conditions, the detection limit for PC_2 was 10-fold higher than for PC_4 (Table 3.1) and, since PC_4 has three thiol groups and PC_2 has only one, PC_4 is more easily detected when using MBrB derivatization. Maier et al. (2003) also reported PCs (~0.02 μmol/g fresh weight) in roots of romaine lettuce grown in control (Cd-free) solution. However, they reported the concentrations in terms of γ-Glu-Cys equivalents; thus, the type of PC in their lettuce was not identified.

In contrast to lettuce, PC_{2-4} were synthesized in the barley root upon chronic exposure to 1.0 μM $CdCl_2$, with the relative amounts of PC_2 being 1-2 orders of magnitude higher than those of PC_3 and PC_4. Similarly, Wang and Wang (2011) found that PC_2 was synthesized quickly as a response to Cd exposure in the marine diatom *Thalassiosira nordenskioeldii* and it was six times higher than PC_3 and PC_4. Sadi et al. (2008) studied Cd^{2+}-PC_n complexes in *Arabidopsis thaliana* and reported Cd^{2+}-PC_2 as the primary complex in wild as well as in genetically modified PC-deficient mutant lines. The high amounts of PCs produced in barley root could have contributed to reduced translocation of Cd to barley shoots relative to lettuce shoots, which in turn could explain why barley leaves appeared healthy after 1 h exposure to 5.0 mM $CdCl_2$ whereas lettuce leaves were visibly negatively affected. Persson et al. (2006) demonstrated the biological importance of Cd^{2+}-PC_n complexation for tolerance towards Cd using two genotypes of barley. They showed that although the total tissue concentration of Cd was similar for both genotypes, the tolerant genotype synthesized significantly more Cd^{2+}-PC_n complexes than the intolerant genotype. Since it is assumed that Cd^{2+}-PC_n complexes transport Cd^{2+} to the root vacuole (Sanità di Toppi and Gabbrielli, 1999) their formation would reduce the amounts of Cd available for translocation to above ground tissues.

The amounts of PCs in barley did not increase further upon exposure to 5.0 mM $CdCl_2$ for 1 h prior to harvest (Table 3.4, Figure 3.2, supplementary data S1 and S2). This could be explained by the substrate availability required for PC synthesis. When plants are exposed to Cd^{2+}, protein degradation provides the amino acids necessary for PC synthesis

(Wu et al., 2004). It is possible that the amino acid pool remained unchanged during the short, 1 h treatment, thus preventing increased production of PCs.

3.4.3 Precursor peptides

Since Cys, γ-EC and GSH are precursors of PC biosynthesis, their amounts are expected to drop (even if only temporarily) upon acute exposure to Cd^{2+}, and might be expected to increase or return to control values under chronic exposure to Cd^{2+} if they are required to supply ongoing synthesis of PCs. In this study, the amounts of γ-EC were either low or below detection limits in all samples and the relative amounts of Cys and GSH varied with both species and tissue type. Roots of both species contained about an order of magnitude less Cys as compared to shoots and there was no consistent response to either chronic or acute exposure to Cd^{2+}. In general, the amount of GSH was reduced in plants grown with chronic exposure to Cd^{2+}. A number of other studies also reported reduced GSH level upon days or weeks of exposure to Cd^{2+} (Scheller et al., 1987; Tukendorf and Rauser, 1990; Lima et al., 2006). However, like PCs, the amounts of GSH returned to control levels in plants given the acute 5.0 mM $CdCl_2$ treatment, indicating that GSH synthesis was rapidly up-regulated, possibly to meet the requirement for PC synthesis or to combat Cd-induced stress. The exception to this was in the lettuce shoots, where the amount of GSH stayed low upon acute exposure to Cd. Other than its role in PC synthesis, GSH is also known to form complexes with Cd^{2+}. Dameron et al. (1989) isolated GSH-coated CdS crystallites in *Candida glabrata* providing direct evidence of biologically formed Cd^{2+}-GSH complexes. Recently Chekmeneva et al. (2011) used isothermal titration calorimetry (ITC) to understand the influence of PC chain length on the Cd^{2+}-PC_n complex stabilities and showed that GSH can form stable Cd^{2+}-GSH complexes at pH 7.5 and 8.5. However, since Cd^{2+}-PC_n complexes are more stable than Cd^{2+}- GSH complexes, it is likely that GSH will play a minor role in detoxifying Cd^{2+} compared to PCs. It has been suggested that GSH might act as a first line of defense against Cd^{2+} toxicity by complexing metal ions before sufficient PCs are synthesized (Thangavel et al., 2007). Once PCs take over the detoxification process, GSH becomes involved in a secondary defense mechanism by scavenging free radicals in Cd^{2+}-induced oxidative stress (Gallego et al., 2005; Rainieri et al., 2005). GSH is not only a precursor

for PC synthesis (Grill et al., 1989) but also an important antioxidant in plants. In the present study, the amount of GSH was always higher in barley, probably contributing to higher chelation of Cd^{2+} compared to lettuce.

3.4.4 Cd^{2+}-PC_n complex formation

Higher concentrations and amounts of PCs and their precursors in barley root compared to lettuce root indicate that the formation of Cd^{2+}-peptide complexes probably contributes to the observed retention of Cd in barley roots. While the calculations of symplastic Cd^{2+} might be overestimates, and some of the PCs in our samples may have degraded, it was determined that there were sufficient PCs in the roots of barley from the chronic 1.0 µM $CdCl_2$ treatment to bind 100% of the putative symplastic Cd^{2+}. If the amounts of Cd^{2+} in the symplast were actually lower than it was predicted and if the concentrations of PCs were actually higher than it was measured, then Cd^{2+}-PC_n complexes could effectively eliminate free Cd^{2+} in the symplast. In barley roots from the acute 5.0 mM $CdCl_2$ treatment, PCs could, in theory, form complexes with only 46% of the symplastic Cd^{2+} but if Cd^{2+} also formed complexes with monothiols then only 15% of the symplastic Cd^{2+} would be predicted to be free ions. In the case of lettuce, PCs were not synthesized but 100% of the total symplastic Cd could theoretically form complexes with the monothiols produced in the roots upon exposure to 1.0 µM $CdCl_2$. The estimated proportion of chelated Cd^{2+} drops to 5% in lettuce roots from the 5.0 mM $CdCl_2$ treatment. However, Cd^{2+}-monothiol formation is not expected to be as efficient as the estimates indicate and the lack of PCs in lettuce roots could explain the higher proportion of total Cd that is translocated to lettuce leaves.

If Sanità di Toppi and Gabbrielli's (1999) model is correct, after Cd^{2+} is released from a PC complex in the vacuole, the PCs could either be degraded by vacuolar hydrolysis or could return back to the cytoplasm. These apo-PCs could serve as a shuttle, bringing more Cd^{2+} into the vacuole. This shuttling process could continue until all the free Cd^{2+} are moved into the vacuole. Based on the estimates calculated in this study, each PC produced in barley roots exposed to the acute, high concentration of Cd would have to carry only 2 Cd^{2+} ions into the vacuole to sequester the amount of Cd estimated in the root symplast. Previously, the role of PCs was thought to be limited to the intracellular

detoxification mechanism by shuttling Cd^{2+}-PC_n complexes into the vacuole. However, recent studies on *Brassica napus* (Mendoza-Cozalt et al., 2008) and *Arabidopsis thaliana* (Gong et al., 2003; Chen et al., 2006) showed that PCs could also play a major role in long-distance transport of Cd^{2+} through xylem and phloem. Mendoza-Cozalt et al. (2008) found that the concentration of PCs was 50 times higher in the phloem sap compared to the xylem sap and concluded that phloem was more active in transporting Cd^{2+} from the source (older leaves) to the sink tissues (root, branches, younger leaves). This seems reasonable because the pH in phloem sap is basic compared to the xylem sap (Shelp, 1987) and would allow greater stability of Cd^{2+}-PC_n complexes. So, it is possible that Cd might be transported within the plant as Cd^{2+}-thiol complexes rather than as free ions. In this study, more PCs as well as their precursors were measured in the shoots of barley compared to lettuce and it is possible that these PCs formed complexes with shoot Cd^{2+} and transported it downwards to the roots. This could be another reason for the observation that a greater proportion of the Cd in barley was found in the root whereas more of the Cd in lettuce was found in the shoot.

3.5 Conclusions

There appears to be a relationship between PC synthesis in the root and Cd translocation to the shoot in barley and lettuce. Between the species, barley had higher concentrations and amounts of PCs and their precursors compared to lettuce and barley retained more Cd in the roots which is equally distributed between apoplast and symplast compartments. However, until direct measures of sub-cellular Cd-distribution and Cd-speciation are available it is difficult to definitively determine the role of PC and its precursors in binding Cd^{2+} in the roots. Further studies are needed to confirm the role of PCs and their precursors in answering the differences in Cd accumulation between barley and lettuce.

3.5.1 Limitations of the study

One of the limitations of this study is the method used to estimate the apoplastic and symplastic Cd within the root. It is unlikely that all of the apoplastic Cd^{2+}, especially in the stele and regions of the cortex near the stele, exchanged with Ca^{2+} ions in the $CaCl_2$ desorption procedure. As well, , some symplastic Cd, especially in the cells near the

epidermis, may have been removed from the cells during the desorption procedure. However, regardless of the chances of either overestimation or underestimation of both the apoplastic and symplastic Cd, the procedure provided an idea of the relative distribution of Cd within the root.

3.6 References

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Chapter 4

4 Localization of Cadmium in Barley and Lettuce Roots by Combining Light Microscopy, Electron Microscopy and X-ray Spectroscopy

4.1 Introduction

The mechanism of Cd tolerance in plants has extensively been studied over the last few decades. In some plants the first line of defense against Cd toxicity is preferential exclusion of Cd^{2+} from active tissues and organs. For example, Ouariti et al. (1997) showed that 98% of total Cd was retained in the roots of *Phaseolus vulgaris* and only 2% was translocated to the shoot; presumably, much of the Cd in the root was in the apoplast or the vacuoles. Pielichowska and Wierzbicka (2004) studied Cd hyperaccumutor *Biscutella laevigata* and found a high concentration of Cd in the trichomes, indicating the Cd that was taken up, translocated and ultimately sequestered in non-metabolic cells. Similar findings were observed in *Arabidopsis thaliana*, which had a high concentration of Cd in the trichomes (Ager et al., 2002; 2003). However, sequestration of Cd in non-active tissues is not always observed. In *Pisum sativum* and *Zea mays*, for example, concentrations from 114 µg/g to 118 µg/g and 21 µg/g to 24 µg/g were detected in the roots and shoots of plants grown in 50 µM Cd^{2+}, respectively. Although there were no differences in these concentrations, *Pisum sativum* accumulated large amounts of lipid peroxidation products inside the root cell in response to Cd and exhibited more severe toxicity symptoms compared to *Zea mays* (Lazano-Rodriguez et al., 1997). The Cd in *Zea mays* was bound to the cell walls, which explained the lack of Cd-stress despite the high concentrations of Cd in the root.

Most studies of Cd distribution within plants measure only bulk tissue (e.g., root, shoot, leaf, stem, etc.) concentrations of Cd. The studies that have examined the cellular and/or subcellular distributions of Cd in plant tissues used a variety of histochemical, imaging and physical fractionation methods. The histochemical methods included using Cd-specific dyes to locate Cd in fresh tissues (Seregin and Ivanov, 1997; Vollenweider et al., 2006; Vieira da Cunha et al., 2008; Hu et al., 2009). These methods detected Cd at the

cellular level; however, it is possible that Cd is redistributed among and within cells during sample preparation, especially if the samples are immersed in an aqueous solution that could cause leaching of Cd. A number of studies used scanning electron microscopy (SEM) or transmission electron microscopy (TEM) along with energy dispersive X-ray spectrometry (EDS, Solís-Domínguez et al., 2007; Hu et al., 2009) or energy dispersive X-ray microanalysis (EDXMA, Rauser and Ackerley, 1987; Wójcik et al., 2005; Van Belleghem et al., 2007; Vazquez et al., 2007; Cocozza et al., 2008) to determine the distribution of Cd in plants. Although these techniques are very powerful, with Cd-specific signals, at low concentrations of Cd^{2+} the proximity of the emission spectra from Ca^{2+} or K^{+} in the sample cannot be excluded. In addition, SEM and TEM usually require dehydration and embedding of the plant sample, which could cause redistribution of Cd during sample preparation. Other imaging techniques include secondary ion mass spectrometry (SIMS, Migeon et al., 2011), microparticle-induced X-ray emission (micro-PIXE, Ager et al., 2002; Vogel-Mikuš et al., 2008), and micro-autoradiography (Cosio et al., 2006). Others have used subcellular fractionation to separate Cd-containing tissues and organelles (Weigel and Jäger, 1980; Wu et al., 2005; Wang et al., 2008); however, the centrifugation, filtering and washing steps might rupture cells or organelles, releasing Cd and redistributing Cd among the fractions.

Other than the techniques mentioned above, micro-synchrotron analytical techniques have also been used to detect Cd at the cellular and sub-cellular levels (Naftel et al., 2001; Hokura et al., 2005; Isaure et al., 2006; Fukuda et al., 2008; Harada et al., 2010; Terada et al., 2010). Micro-synchrotron X-ray fluorescence (μ-XRF) has a micron-scale beam size and highly sensitive detection systems that can provide spatially resolved concentration maps of elements within single plant cells. When added to micro X-ray absorption near-edge structure (μ-XANES) and micro X-ray absorption fine structure (μ-XAFS) spectroscopy, the system can also provide information on element-specific oxidation state(s) and coordination environment(s) of metals inside the cell.

Although the techniques mentioned above provided insight into the Cd distribution at the cellular and subcellular levels, the findings are not consistent. For example, some studies reported either no (*Agrostis gigantean* and *Zea mays* grown in 3.0 mmol/m^3 Cd^{2+} for 4 d,

Rauser and Ackerley, 1987; *Phaseolus vulgaris* grown in 0.5 µM Cd^{2+} for 6 d, Vázquez et al., 1992; *Allium cepa* exposed to 10 mM Cd^{2+} for 72 h, Liu and Kottke, 2004) or very low concentrations of Cd in the cell wall (*Phaseolus vulgaris* grown in 0.45 mM Cd^{2+} till the development of first trifoliate leaves, Weigel and Jäger, 1980), while others found the cell wall to be a very important site for binding Cd at the cellular level (*Hordeum vulgare* grown in 5 µM Cd^{2+} for 25 d, Wu et al., 2005; *Lupinus albus* grown in 150 µM Cd^{2+} for 35 d, Vázquez et al., 2007; *Bechmeria nivea* grown in 7 mM Cd^{2+} for 20 d, Wang et al., 2008). These differences could be due to differences among plant species and Cd^{2+} concentrations used in the studies as well as the different sample preparation and Cd-detection methods used. Using multiple techniques on samples taken from individual plants and growing two species under the same conditions could solve the problem and provide a more consistent answer to the question of where is Cd localized within the plant.

Most of the studies mentioned above studied Cd distribution at the cellular and sub-cellular levels of hyperaccumulator plants, which can grow and accumulate high concentrations of Cd (> 100 µg/g leaf dry weight, Bert et al., 2002) in their aboveground biomass without showing visible toxicity symptoms. Those studies have provided useful information on qualitative imaging of cellular and sub-cellular Cd and, in a few cases, Cd^{2+} speciation, and have also expanded our current understanding of the mechanisms of Cd accumulation in hyperaccumulator plants. However, low metal-accumulating plants (such as agricultural crops) are expected to use different mechanisms in regulating their intracellular concentrations of Cd since lesser amounts of Cd tend to be taken up by these plants. As per knowledge, only a few studies have been conducted on the Cd distribution in agricultural crops (Weigel and Jäger, 1980; Rauser and Ackerley, 1987; Seregin and Ivanov, 1997; Naftel et al., 2001; Vieira da Cunha et al., 2008; Terada et al., 2010); however, none of these studies provided information on Cd speciation in the plants.

In the present study, multiple techniques including histochemical staining, SEM-WDS and µ-XRF were used to investigate the distribution and coordination environment of Cd in lettuce (*L. sativa*) and barley (*H. vulgare*) roots. The plants were chosen because in previous experiments (Chapter 2 and Chapter 3), I found approximately 80% of the total

Cd was translocated to leaves of lettuce, whereas only 20% of the total Cd was translocated to barley leaves. This led to the hypothesis that barley and lettuce have different mechanism(s) to either store Cd in the root or translocate Cd to the leaves. Information about the distribution of Cd in the roots will determine the proportion of Cd bound to the cell walls and the in the vacuoles, whereas information about the co-ordination environment will indicate to which class of molecules the Cd^{2+} is bound.

4.2 Methods and Materials

4.2.1 Germination and growth conditions

Lettuce (*L. sativa* L. cv. Grand Rapids) and barley (*H. vulgare* L. cv. CDC McGwire, hulless 2-row feed barley) seeds were germinated on moist (RO water) filter paper in the dark at room temperature. Seedlings were transferred to sand-filled pots (15 cm diameter) when the radicles were approximately 1 cm long (24-36 h) and kept in a growth chamber set to 21°C, 187 ± 2 μmol/m^2/s, 60% relative humidity, and a 16 h day length. Potted seedlings were watered with nutrient solution (pH 6.0) consisting of 1.0 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1.0 mM K_2HPO_4, 0.40 mM KNO_3, 0.30 mM $Mg(NO_3)_2 \cdot 6H_2O$, 0.30 mM NH_4NO_3, 0.10 mM K_2SO_4, 10.0 μM $FeCl_3 \cdot 6H_2O$, 10.0 μM Na_2EDTA, 6.0 μM H_3BO_3, 2.0 μM $MnCl_2 \cdot 4H_2O$, 0.50 μM $ZnSO_4 \cdot 7H_2O$, 0.15 μM $CuSO_4 \cdot 5H_2O$ and 0.10 μM Na_2MoO_4. After 7 days in sand culture the seedlings were transferred to hydroponic solutions in 1.4 L glass jars. The jars were filled with nutrient solution (pH 6.0) to which either 0 or 1.0 μM $CdCl_2$ was added. A total of three replicates were used in each treatment. In each jar, one seedling was suspended with a 0.5 × 1 × 6 cm piece of foam secured in the lid. The sides of the jars were covered with black cloth to prevent algal growth. The jars were connected to an aeration system and the plants were provided with fresh nutrient solution including the corresponding Cd treatment every second day.

On the 28th day in hydroponic culture, the lettuce and barley seedlings from the 1.0 μM $CdCl_2$ treatments were transferred into fresh nutrient solution (pH 6.0) with 5.0 mM $CdCl_2$ and 10.0 mM $CdCl_2$, respectively for 1 h. A comparatively higher concentration was selected for barley seedlings since they could accumulate higher amounts of Cd compared to lettuce. At harvest, fresh weights of roots and shoots were recorded, rinsed

in RO water and 1.0 g of a subsample of root was immediately fixed in 2% glutaraldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) overnight before preparing for microscopic analysis. The remainder of the root and shoot samples were oven dried (60°C) to constant weight and stored for Cd analysis.

4.2.2 Cd content

The concentrations of Cd in samples were determined following the method in section 2.2.4.

4.2.3 Procedures for microscopic study

4.2.3.1 Light microscopy

The localization of Cd in root tissues was studied using the histochemical method developed by Seregin and Ivanov (1997), which involves staining with dithizone, a reagent that produces a reddish compound (insoluble red salt, Cd-dithizonate) in the presence of Cd. At first, 3.0 mg dithizone (Sigma-Aldrich, MO, USA) was dissolved in 6.0 mL of acetone (Caledon, Georgetown, ON, Canada). Then 2.0 mL distilled water and 1 drop of glacial acetic acid (EM Science, Gibbstown, NJ, USA) were added to the solution because the reaction is Cd-specific in a weakly acidic medium (Seregin and Ivanov, 1997). Once the dithizone solution was prepared, root cross sections were prepared (hand sectioned) from the roots that had been fixed in glutaraldehyde. The sections were taken from the region above the root tip where the cellular distribution of Cd was clearly visible. At the root tip, the concentration of Cd was very high due to the absence of endodermis and vascular tissues. The sections were put on a glass slide with 2-3 drops of dithizone solution, covered by a cover slip and immediately examined under a light microscope.

4.2.3.2 Scanning electron microscopy assisted with wavelength dispersive spectrometry (SEM-WDS)

4.2.3.2.1 Dehydration and embedding

Root samples were prepared for SEM following the sample preparation method developed in the Geomicrobiology Laboratory, Department of Earth Science, Western

University. Roots were taken out of glutaraldehyde solution, rinsed in RO water and cut into small pieces (1 cm in length). The sections were dehydrated in an ascending series of 25%, 50%, 75%, 100%, 100%, 100% acetone with 15 min incubation at each step. After dehydration, the roots were gradually saturated with an epoxy resin mixture consisting of three components: embed 812 (EMS, Hatfield, PA, USA), dodecenyl succinic anhydride (DDSA, EMS, Hatfield, PA, USA) and nadic methyl anhydride (NMA, EMS, Hatfield, PA, USA) in a ratio of 20:9:10. The components were mixed with a vortex to ensure even distribution of plastics in the mixture. This was very important since uneven hardness in the final product might result in dragging scratches during cutting, polishing and coating. The acetone was replaced with epoxy resin during a gradual incubation series consisting of [50% acetone: 50% epoxy], [25% acetone: 75% epoxy] and [10% acetone: 90% epoxy] with a 1 h incubation at each step or until the sample sunk to the bottom. After that, the sections were left overnight in 100% epoxy to ensure complete impregnation of resin inside the root. On the following day, the sections were embedded in a fresh batch of epoxy consisting of embed 812, DDSA, NMA and (2, 4, 6-{Tri(Dimethylaminoethyl) phenol} (DMP-30, EMS, Hatfield, PA, USA) in a ratio of 20:9:12:0.72 and kept at 60°C for 48 hours for hardening.

4.2.3.2.2 Polishing and coating

The resin-embedded root samples were polished using a circular metallographic wet paper grinding wheel (60 grit/500 grit). At first, the samples were cut using a diamond saw to create a flat surface. Then the samples were polished, starting with carborundum paper and ending with diamond paste. The flat surface is critical for good quantitative analysis since a scratched sample surface can lead to uneven production of signals and can produce erroneous results. After polishing, the samples were coated with osmium tetroxide (OsO_4, 5 nm), which produces an electrically conductive surface and thus prevents charging under the electron beam.

4.2.3.2.3 SEM-WDS analysis

The coated samples were mounted on carbon stubs using carbon paint prior to SEM analysis. Carbon has low atomic contrast in the backscattered mode, where samples

mounted on the surface can be isolated easily from any background effects. Carbon atomic contrast appears black in backscatter mode, which is very useful in imaging. The roots were targeted using secondary electron (SE) and backscatter (BSE) imaging and the images were used to generate Cd distribution maps in wavelength dispersive spectrometry (WDS) set to 15 kV. The cellular (epidermis, cortex, endodermis and vascular bundle) and sub-cellular (cell wall or "apoplast" and inside the cell or "symplast") quantification of Cd was conducted from point analysis of Cd^{2+} signals (counts per second, cps) generated across the root cross-section. A total of three line scans were run from three different roots (experimental replicates) and the signals from each of the epidermis, cortex, and endodermis, vascular tissue, apoplast and symplast regions were averaged and used as the reported WDS signal.

4.2.3.3 Micro X-ray fluorescence (µ-XRF) spectroscopy

Thin cross sections of approximately 200 µm thickness were prepared from resin-embedded roots. Similar to light microscopy, the sections were taken from the zone immediately above the root tip. Samples were mounted onto rinzyl plastic micro slides using double-sided carbon tape (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). The experimental set-up followed that of Fukuda et al. (2008). The incident beam was monochromatized by a Si 111 monochromator to 37 keV in order to excite the k-lines of Cd^{2+} and to minimize overlap of the k-line peak with the Compton scattering peak. The high flux beam (3.7 × 1011 photon/sec at 37 keV) helped to generate measurement over a shorter period of time. The incident beam was focused down to 2 µm × 2 µm (or less) using a set of Kirkpatrick-Baez (K-B) mirrors. Spatially-resolved µ-XRF analyses were conducted over 500 µm × 500 µm regions of the samples, which were mounted 45 degrees to the incident beam. The fluorescence X-rays were measured using a Canberra 16-element Ge detector. The fluorescence X-ray intensity was normalized by the intensity of the incident X-ray beam to produce a two-dimensional Cd map.

4.2.4 Statistical analyses

SigmaPlot (version 11.0) was used for statistical analyses and graphics. One-way ANOVA was used to detect treatment effects and Tukey's test was used to determine significant differences between treatment means ($P<0.05$).

4.3 Results

4.3.1 Cd uptake

The concentration of Cd was below the detection limit in the plants grown in 0 µM Cd, except for roots of barley, where the concentration was just above the detection limit (Table 4.1). After lettuce and barley roots were exposed to 5.0 mM Cd and 10.0 mM Cd, respectively, for 1 h prior to harvest, the concentrations of Cd in the roots and shoots increased, with roots having 70 to 213-fold higher concentrations than the shoots. The pattern was similar for the total amount of Cd (amount = Cd concentration × biomass); amounts of Cd increased in response to the higher Cd treatments and roots retained 15-fold and 51-fold more Cd than the shoots in lettuce and barley, respectively. Regardless of differences in Cd concentrations, lettuce translocated 2.4-fold more Cd to the shoot than did barley (Table 4.1).

4.3.2 Cd localization in root tissues

4.3.2.1 Root uptake

The red stain in root samples was attributed to Cd^{2+}-dithizone by comparing the control roots to the Cd-treated roots (Figures 4.1 A, B; 4.2 A, B). In every case, reddish coloured complexes were found only in Cd-treated tissues (personal observation).

Cd was detected in the root hairs of both lettuce (Fig 4.1B) and barley (Fig 4.2 B). The intensity was very high at the root tip, where dithizone developed a strong reddish colour, indicating the presence of Cd. A surface view of intact roots stained with dithizone showed distribution of Cd along the root length with higher concentration of Cd in barley (Figure 4.2 C) compared to lettuce (Figure 4.1 C).

Table 4.1: Concentration and amount of Cd in lettuce and barley grown in different $CdCl_2$ treatments

Species	Treatment (mM $CdCl_2$)	Root Cd (mg/g)		Shoot Cd (mg/g)		Root Cd (mg)		Shoot Cd (mg)	
Lettuce	0	<dl [a]		<dl [a]		<dl [a]		<dl [a]	
	5	19.156 (0.052) [b]		0.261 (0.009) [b]		3.741 (0.303) [b]		0.253 (0.018) [b]	
	One-way ANOVA	p	$F_{(1,5)}$	p	$F_{(1,5)}$	p	$F_{(1,5)}$	p	$F_{(1,5)}$
		0.001	1159.84	0.001	683.16	0.001	152.40	0.001	189.01
Barley	0	0.004 (0.003) [a]		<dl [a]		0.003 (0.002) [a]		<dl [a]	
	10	13.675(2.038) [b]		0.064 (0.006) [b]		5.356 (1.543) [b]		0.105 (0.018) [b]	
	One-way ANOVA	p	$F_{(1,5)}$	p	$F_{(1,5)}$	p	$F_{(1,5)}$	p	$F_{(1,5)}$
		0.01	45.96	0.001	102.70	0.02	11.99	0.01	33.81

Plants were grown with 0 or 1.0 µM $CdCl_2$ for 28 d. The plants grown with Cd were transferred to either 5.0 mM $CdCl_2$ (lettuce) or 10.0 mM $CdCl_2$ (barley) for 1 h immediately prior to harvest. Within each tissue, different lower case letters indicate significant differences in Cd concentration and Cd accumulation, as determined by post-hoc Tukey tests. Values are mean (SE), n=3 for each treatment, dl = detection limit.

4.3.2.2 Cellular and subcellular distribution

Roots from plants grown in the control treatment had no Cd-staining (Figures 4.1 A; 4.2 A), whereas Cd was detected in all the tissues of Cd treated roots (Figures 4.1 D-F; 4.2 D-F). However, intensity of staining varied with the species and tissue types. The darkest Cd-staining was observed in the stele followed by the epidermis and cortex in barley (Figure 4.2 D). Inside the cortex, Cd was detected both in the apoplast (cell wall) and symplast (inside the cell), and the stain intensity in these two compartments was similar (Figure 4.2 E). Inside the stele, Cd was detected throughout the vascular cylinder with the highest stain intensity in the endodermis and cell walls of the xylem (Figure 4.2 F). This pattern was consistent with the Cd-distribution maps generated by SEM-WDS (Figure 4.2 G) and µ-XRF (Figure 4.2 H). In both cases, very high concentrations of Cd were observed in the endodermis, followed by the epidermis, cortex and vascular bundle (Figure 4.2 G, H). Whereas the concentrations of Cd in the apoplast and symplast seemed equal in the cortical tissues, the concentrations in the vascular parenchyma cells appeared higher compared to the xylem vessels in the vascular bundle (Figure 4.2 G, H).

Along with generating Cd distribution maps, WDS also provided quantitative information about the distribution of Cd in the roots (Figure 4.3 C, D). Similar to the imaging analysis, the signals of Cd were the highest in the endodermis (Figure 4.3 C) and there were no differences between apoplast and symplast in the cortex (Figure 4.3 D). The signals of Cd in the epidermis, cortex and vascular bundle were 5-fold lower compared to the endodermis.

In contrast to barley, there were no differences in the intensity of Cd staining among the cortex, endodermis and vascular bundle (Figure 4.1 D) in lettuce; however, detailed imaging of the cortex showed a higher intensity of Cd staining in the apoplast compared to the symplast (Figure 4.1 E). This result was consistent with the Cd distribution maps generated by SEM-WDS (Figure 4.1 G) and µ-XRF (Figure 4.1 H) and was different from the quantitative analysis based on WDS. WDS showed no differences in Cd signals among the tissues of epidermis, cortex, endodermis and vascular bundle (Figure 4.3 A); however, similar to the imaging analyses, the concentration of Cd was higher in the

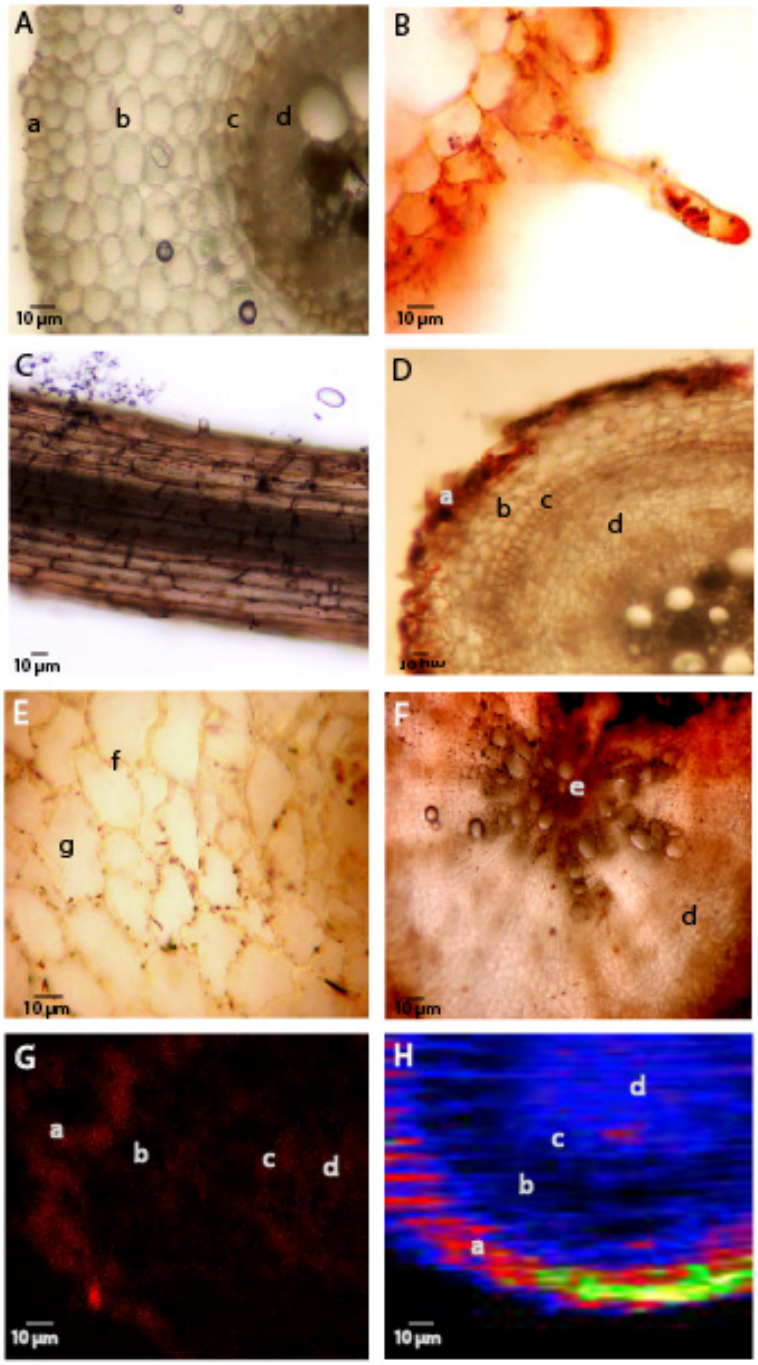

Figure 4.1: Localization of Cd in the tissues and cells of lettuce.
Within the figure: (A) root cross section (c.s.), without Cd treatment (control), (B) root hair, (C) root surface, (D) root c.s., with Cd treatment, (E) cortical cells, (F) vascular cells, (G) root distribution of Cd, SEM-WDS map, (H) root distribution of Cd, micro-XRF map. Different regions in the root are: (a) epidermis, (b) cortex, (c) endodermis, (d) vascular region, (e) xylem, (f) apoplast and (g) symplast. Within A-G, red indicates high concentration of Cd in the root; In H, yellow, green, red, blue and black indicate comparatively higher to lower concentrations of Cd within the root .

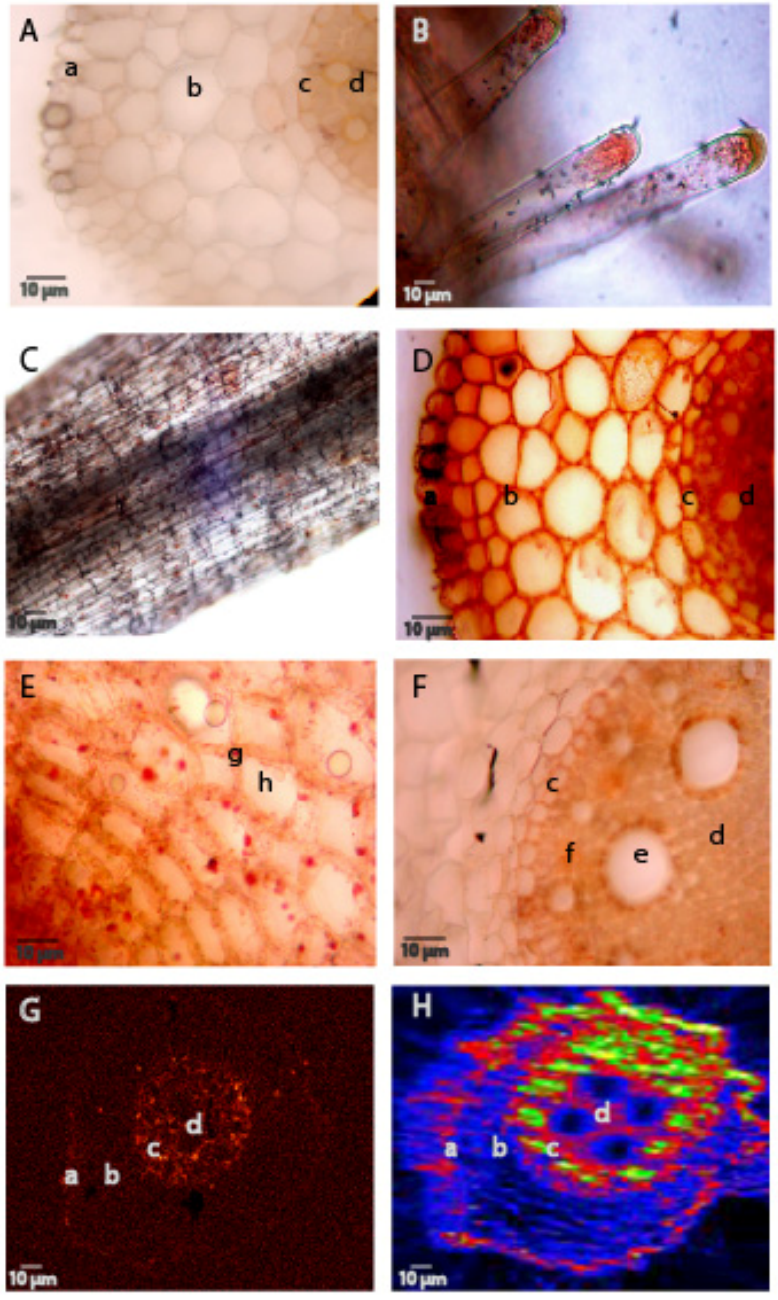

Figure 4.2: Localization of Cd in the tissues and cells of barley.
Within the figure: (A) root c.s., without Cd treatment (control), (B) root hair, (C) root surface, (D) root c.s., with Cd treatment, (E) cortical cells, (F) vascular cells, (G) root distribution of Cd, SEM-WDS map, (H) root distribution of Cd, micro-XRF map. Different regions in the root are: (a) epidermis, (b) cortex, (c) endodermis, (d) vascular region, (e) xylem, (f) phloem, (g) apoplast and (h) symplast. Within A-G, red indicates high concentration of Cd in the root; In H, yellow, green, red, blue and black indicate comparatively higher to lower concentrations of Cd within the root.

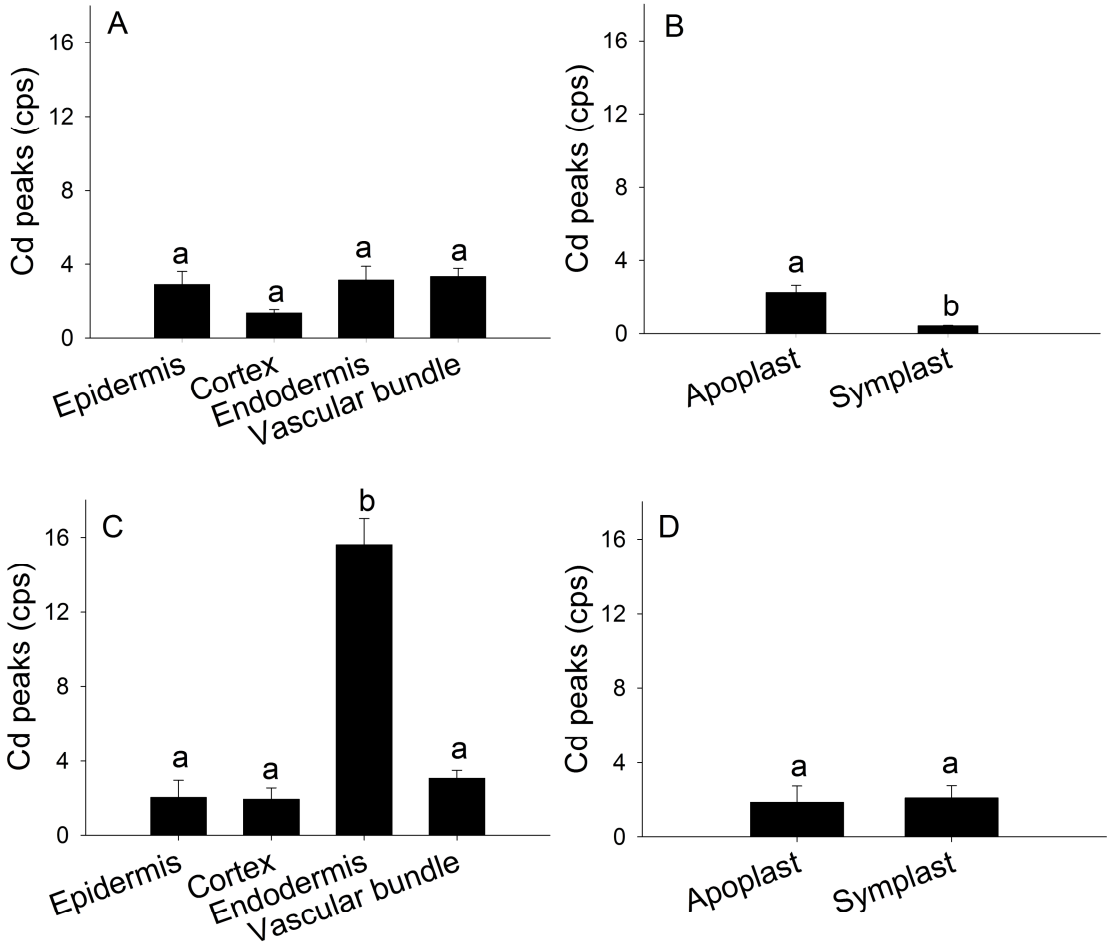

Figure 4.3: Intensity of Cd (counts per second, cps, as generated by WDS) in different tissues (left panels) and subcellular regions (right panels) of lettuce (top panels) and barley (bottom panels) roots

The plants were grown in nutrient solution supplemented with 1.0 µM $CdCl_2$ for 28 days and then exposed to either 5.0 mM (lettuce) or 10.0 mM (barley) $CdCl_2$ for 1 h prior to harvest. Relative Cd in lettuce (A) root tissues and (B) apoplast and symplast within the cortex, and barley (C) root tissues, and (D) apoplast and symplastic within the cortex. Within the species, different lower case letters indicate significant differences in Cd accumulation, as determined by post-hoc Tukey tests. Values are mean (+ SE), n=3 for each region.

apoplast compared to symplast (Fig 4.3 B).

4.4 Discussion

4.4.1 Distribution of Cd between roots and shoots

The plant species showed differences in their ability to retain Cd in the roots. Regardless of the fact that barley was exposed to a 2-fold higher concentration of Cd compared to lettuce in the hour prior to harvest, lettuce translocated a 2.4-fold higher amount of Cd to the shoot compared to barley. This result clearly showed the ability of barley to retain Cd in the root. In the present study, barley retained 98% of the total plant Cd in the root and translocated the rest to the shoot. On the other hand, lettuce retained 94% of total Cd in the root and translocated 6% to the shoot. The proportions of total Cd retained in the roots were consistent with the initial findings of distribution of Cd in lettuce and barley exposed for 1 h to a high concentration of Cd in Chapter three.

In the previous Chapters, the plants took up measurable amounts of Cd in the roots and shoots without showing toxicity symptoms at 1 µM Cd. In the present study, plants were grown in 1.0 µM Cd for 28 days and then exposed to 5.0 mM Cd (lettuce) or 10.0 mM Cd (barley) for 1 h prior to harvest. At the end of the hour, lettuce seedlings were wilted and were likely unable to continue regular physiological activities due to the toxic effects from the accumulation of high amounts of Cd in the shoot. On the other hand, barley seedlings remained turgid at the end of the hour of exposure to 10.0 mM Cd, again showing higher tolerance against Cd toxicity compared to lettuce. However, , 1 h was not enough time to redistribute Cd from the roots to the shoot and most (98%) of the Cd taken up by barley was retained in the root.

4.4.2 Cellular and subcellular localization of Cd in the root

There were clear differences in the localization of Cd between lettuce and barley. In barley, the intensity of Cd was the highest in the endodermis, intermediate in vascular bundle and the lowest in the epidermis and cortex. This pattern was consistent among the three different techniques used and also between the imaging and semi-quantitative WDS analysis. In the case of lettuce, imaging analyses detected higher intensities of Cd in the

epidermis, endodermis and vascular bundle compared to the cortex. However, when the signals of Cd were quantified in WDS, there were no differences among the signals of Cd and that Cd was evenly distributed from the epidermis to the stele (Figure 4.3 A).

By comparing the intensity of Cd signals in different tissues in lettuce and barley it is possible to propose that the difference in the ability of these two species to translocate Cd to the shoot depends on their ability to immobilize Cd in the root. Barley formed a major barrier against Cd movement in the endodermis. The very high intensity of Cd signals measured in the endodermis in all three techniques used clearly demonstrates the ability of these tissues to bind Cd in the root. In barley, the Casparian band at the endodermis was a likely barrier to further movement of Cd in the apoplast. The increased accumulation of Cd at this barrier indicates that relatively little Cd was transported into the symplast for subsequent movement across the endodermis. Since there were no differences in the intensities of Cd signals among different tissues in lettuce root, it is possible that Cd encountered less effective barriers to movement in the lettuce root and subsequently was translocated to the shoot. Either the Casaparian band of lettuce was more permeable to Cd than that of barley or proportionately more Cd was transported into the symplast in lettuce.

4.4.2.1 Epidermis

The epidermis provided the first barrier to Cd accumulation, preventing Cd from entering the root, and as a result Cd accumulated in this region. Other studies also reported a high concentration of Cd in the epidermis (Küpper et al., 2000; Van Belleghem et al., 2007; Hu et al., 2009). Küpper et al. (2000) studied the distribution of Cd in the roots of hydroponically grown *Arabidopsis halleri* and found cadmium phosphate precipitates ($Cd_3(PO_4)_2$) in the epidermis. This observation seems reasonable under this experimental condition, where seedlings were grown in aerated nutrient solution similar as Küpper et al. (2000); however, this seems unlikely under natural condition where depletion of phosphorus (P) rather than accumulation is expected (Marschner, 1995). Van Belleghem et al. (2007) examined the subcellular distribution of Cd of the epidermal cells of the roots of *Arabidopsis thaliana* and found high concentrations of P and sulphur (S) in the

cell wall and cytoplasm, respectively. It is possible that the species formed Cd^{2+}-S and Cd^{2+}-P complexes and retained Cd in the vacuoles of the epidermal cellss.

Between the epidermis and cortex, barley has an additional layer of cells called the exodermis (Gierth et al., 1999). Similar to the endodermis, exodermal cells have a Casperian strip consisting of pectin and suberin that can block the radial flow of water, nutrients and other ions (Gierth et al., 1999). The high concentration of Cd observed between the epidermis and cortex in barley roots might be due to accumulation of Cd in the exodermis and reduced radial movement of Cd towards the stele. Lettuce does not have an exodermis in the root, which menas there would be fewer barriers to Cd movement within the root compared to barley.

4.4.2.2 Cortex

The concentration of Cd was lower in the cortex in both lettuce and barley compared to other tissues in the root; however, considering the largest proportion of root volume is made up of the cortex it is possible that it might play an important role in retaining Cd in the root. The detail images from Cd-dithizone stain, SEM-WDS and μXRF, as well as the quantitative analysis using SEM-WDS, showed that the species differed in the distribution of Cd in cortex. While the concentration was 5.5-fold higher in the apoplast of cortex compared to symplast in lettuce, there were no differences in Cd signal between apoplast and symplast in barley. This result was consistent with the findings from Chapter three, where lettuce and barley were exposed to 5.0 mM Cd for 1 h followed by desorption of apoplastic Cd^{2+} using $CaCl_2$. Since the apoplast is the cell wall compartment of a cell, it consists of polysaccharides, including cellulose and hemicellulose, as well as pectin and proteins. These polysaccharides and proteins have hydroxyl, carboxyl, amino and aldehyde groups which can bind Cd^{2+}. However, compared to symplastic Cd, apoplastic Cd is loosely bound and can be available for translocation to the shoot.

In contrast to apoplastic Cd, symplastic Cd is more immobile since the Cd^{2+} ions form complexes with chelators (for example, organic acids, phytochelatins, PC and their precursor peptides; please see Chapter three, section 3.4, for details about PC-mediated

Cd^{2+} detoxification mechanisms), which either precipitate in the cytoplasm or move into the vacuole. Since barley has half of its cortical Cd in the symplast, it is possible that this Cd^{2+} would form complexes with PC and other S-containing compounds and stay in the root. Other studies also reported symplastic Cd in the roots of a number of species including *Arabidopsis thaliana* (Van Belleghem et al., 2007) and *Echinochloa polystachyas* (Solís-Domínguez et al., 2007).

4.4.2.3 Endodermis

The concentration of Cd was very high in the endodermis compared to other tissues in barley root. Restricted movement of ions from the cortex into the stele is expected due to the presence of the casperian strip, which is made of suberin, and blocks the apoplastic movement of ions including Cd^{2+} (Tester and Leigh, 2001). The only pathway into the stele is via the symplast of the endodermis. A number of other studies also reported a high concentration of Cd in the endodermis (Wojcik et al., 2005; Van Belleghem et al., 2007; Terada et al., 2010).

It was surprising to find no differences in the concentration of Cd among the tissues in lettuce root. This means that, while the radial flow of Cd^{2+} was blocked at the endodermis of barley, the movement of Cd^{2+} was not restricted in the root of lettuce. It is possible that Cd^{2+} was not transported into the symplast of the barley in the cortex or at the endodermis, or that the symplastic Cd formed complexes with PCs and was deposited in the vacuole, preventing movement towards the stele. However, since no PCs were detected in the lettuce root (Chapter three), it is possible that Cd^{2+} in the symplast of the endodermis was free to move towards the stele. This idea is consistent with Yamaguchi et al. (2011), who studied root-to-shoot translocation of Cd in two contrasting species of *Solanum* that differed in their ability to translocate Cd to the shoot. They found that the low Cd translocating species accumulated a high amount of Cd in the endodermis and was unable to load Cd to the xylem.

4.4.2.4 Vascular bundle

Once Cd crosses all the dermal barriers and reaches the vascular bundle, it can again bind to the vascular tissues before loading to the xylem vessels (Isaure et al., 2006; Van

Belleghem et al., 2007; Hu et al., 2009). This was observed in the present study, where high intensities of Cd were detected in the xylem parenchyma and cell walls of xylem vessels (Figures 4.1 F; 4.2 F). However, the ability to load Cd^{2+} from the xylem parenchyma to the xylem vessels depends on the activity of transporters (Mori et al., 2009). Recently, Yamaguchi et al. (2011) identified a xylem-loading citrate transporter, AtFRD3, which was down-regulated during the process of Cd acclimation in *Solanum torvum* grown in 0.1µM Cd. It is possible that lettuce might have those, or similar, transport proteins in the vascular bundles and loaded Cd^{2+} from the xylem parenchyma to the xylem vessels; however, this needs confirmation.

Along with the xylem, Cd was also detected in the phloem in barley (Figure 4.2 F). It is possible that Cd was redistributed from the shoot down to the root in the phloem as part of the Cd detoxification process in barley. Phloem-mediated Cd redistribution has been reported in *A. thaliana* (Van Belleghem et al., 2007) and *Triticum aestivum* (Cakmak et al., 2000). Cakmak et al. (2000) applied ^{109}Cd in the leaves of wheat and identified Cd in the root that was redistributed from the shoot. Mendoza-Cozatl et al. (2008) identified high levels of PCs, glutathione (GSH) and Cd in the phloem sap of *Brassica napus* and suggested that, along with xylem Cd transport, the phloem is a major vascular system for long-distance source to sink transport of Cd^{2+} as Cd^{2+}-PC and Cd^{2+}-GSH complexes. Since lettuce has fewer PCs in the shoot compared to root (Chapter three), it is possible that less Cd was redistributed in lettuce root compared to barley.

4.5 Conclusions

The results clearly indicated that Cd was blocked by more effective barriers in barley compared to lettuce along the route to the xylem vessels and subsequent translocation to the shoot. A major barrier appears to be related to cells that have a Capsparian band, but it is also reasonable to suggest that Cd^{2+} is more readily transported across membranes in lettuce. Moreover, a higher concentration of Cd was detected in the symplast of barley, which indicates that barley might immobilize more Cd via chelation in the root compared to lettuce, which would reduce transfer to the shoot.

4.5.1 Limitations of the study

There are chances that Cd was redistributed among and within cells during sample preparation, especially when the root-sections were immersed in dithizone (an aqueous solution) that could cause leaching of Cd. In the case of SEM and x-ray fluorescence microscopy, the roots were dehydrated in acetone and then embedded in epoxy before polishing and coating for SEM-WDS analysis. The root samples were fixed in 2% glutaraldehyde at harvest; however, the chances of Cd redistribution during these sample preparation steps can not be eliminated.

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Chapter 5

5 General Discussion and Future Work

5.1 General Discussion

The research interests of this study are focused on metal-contamination in plants and it is expected that the results of the experiments can be used to ensure that the metal content of edible plants is kept to a minimum.

Since Cd dissolves readily in water, water-related Cd accumulation in lettuce, barley and radish was investigated as a beginning approach. Transpiration-related Cd accumulation in these three species was studied by growing them in various Cd^{2+} concentrations, ranging from 0 to 2 µM Cd^{2+} (Chapter two). Regardless of species, plant Cd content increased with increased Cd^{2+} concentration in the nutrient solution and, while there was a positive relationship between Cd content and total volume of water transpired, the strength of the relationship was species-specific. Among the species, radish took up the least amount of Cd and transpired the highest amount of water. On the other hand, barley took up the highest amount of Cd and transpired an equal amount of water compared to lettuce. Among the species, lettuce showed the strongest correlation between shoot Cd and the total amount of water transpired per unit leaf area. This result clearly indicates that that transpiration-related Cd accumulation varies among the studied species. The traits that might contribute to more or less transpiration in the studied species are shown in Figure 5.1 and 5.2. These include manipulating osmotic gradient, stomatal conductivity, etc. (Fig. 5.1 A, Fig. 5.2 A)

Although all three species showed a positive correlation between transpiration and Cd accumulation, Cd^{2+} budgeting showed that the plants accumulated more Cd than was available through water uptake. So, it is clear that transpiration alone can not explain Cd accumulation in the studied species and it is possible that, along with transpiration, active uptake of Cd^{2+} might also be occurring. A number of studies reported energy-dependent Cd^{2+} uptake in plants through Ca^{2+} (Zhao et al., 2002), Zn^{2+} (Ueno et al., 2008), and Fe^{2+} (Nakanishi et al., 2006) channels and possibly by Cd^{2+} transporters (Lombi et al.,

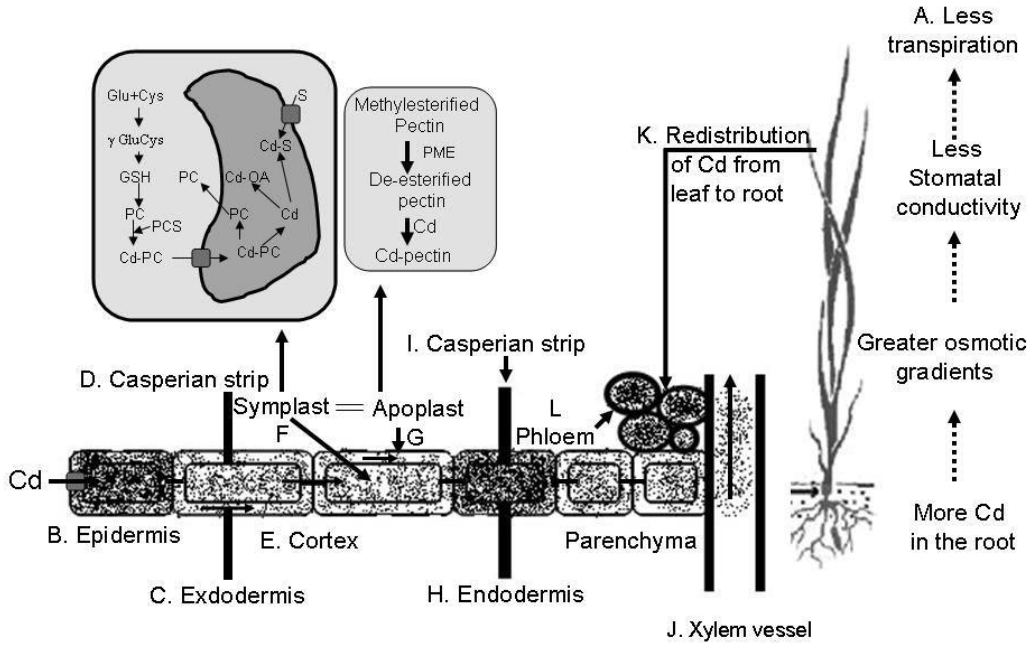

Figure 5.1: Mechanisms of Cd accumulation and distribution in barley

(see text for full explanation)

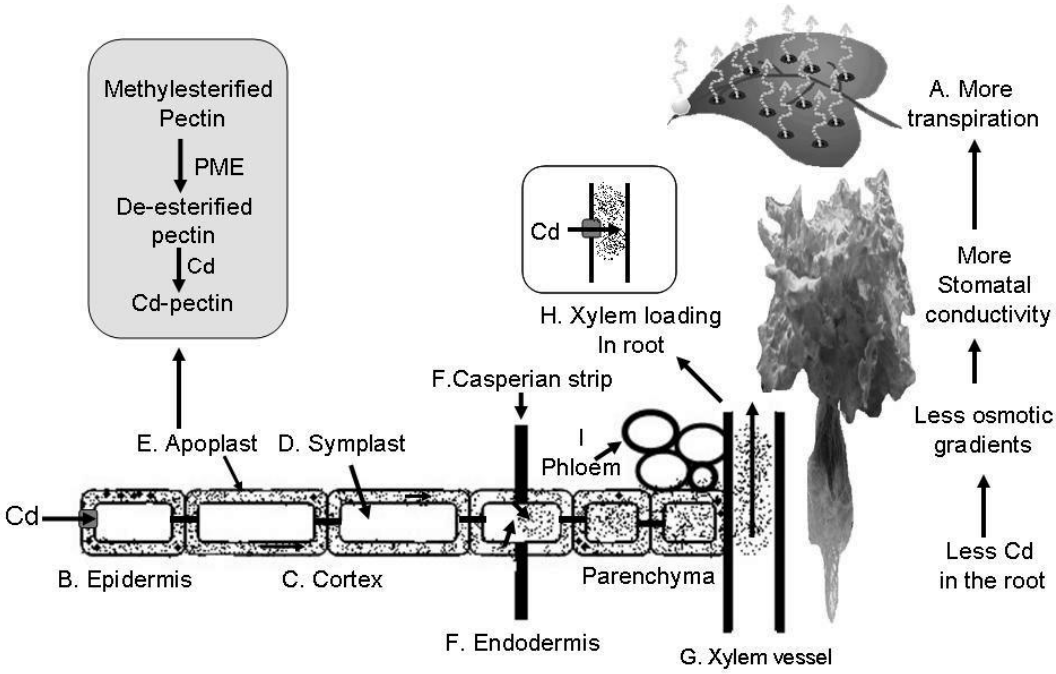

Figure 5.2: Mechanisms of Cd accumulation and distribution in lettuce

(see text for full explanation)

2001).These transporters are expected to be present in the epidermis of the root of both barley and lettuce (Fig 5.1 B and 5.2 B) as well as in the membranes of cortical cells (Fig 5.1 C and Fig 5.2 C). Based on the results of Chapter 4, it is predicted that the density of such transporters are higher in endodermal cells (Fig 5.1 E and Fig 5.2 E) of lettuce as compared to barley..

Another interesting finding from the transpiration study was that, although less total Cd was measured in lettuce plants (root plus shoot) compared to the other two species, lettuce shoots contained higher amounts of Cd than were measured in shoots of other two species. While barley and radish retained 80% and 44%, respectively, of the total plant Cd in the root, lettuce had only 15% of the total plant Cd in the root. It is possible that barley and radish have mechanisms to restrict Cd^{2+} in their roots that resulted in reduced Cd^{2+} translocation to the shoot. Specifically, the different species may accumulate Cd in different compartments within the root. For example, whereas some studies reported root Cd to be mainly apoplastic (Vázquez et al., 2007; Wang et al., 2008), others found Cd^{2+} mostly bound within the symplast (Rauser and Ackerley, 1987; Vázquez et al., 1992). In the present study, whereas barley accumulated Cd both in the apoplast and symplastof the root , lettuce had higher amounts of Cd in the apoplast in the root). Within the symplast Cd could be sequestered in the vacuoles of roots cells via PC-mediated chelation (Salt and Rauser, 1995; Fig 5.1 F). Apoplastic Cd would have a different fate and it is possible that Cd^{2+} might form complexes with polysaccharides (cellulose, hemicellose and lignin) and proteins present in the cell wall (Fig 5.1 G and Fig 5.2 E). In addition, the exodermal layer in barley roots provides an added location at which Cd ions might accumulate. Based on the distribution of Cd in the roots and shoots of lettuce and barley in Chapter two, apoplastic and symplastic distribution of Cd in the roots were measured and the latter was related to PC-mediated Cd^{2+} restriction in the roots in Chapter three. Since radish had very little Cd in the root and was considered to have low risk of toxicity for consumption when grown in the concentrations tested, it was not included in subsequent studies.

In Chapter three (PC study), lettuce and barley were grown with a chronic, low concentration of Cd^{2+} (1.0 µM) and tested for apoplastic and symplastic distribution of

Cd in the root. However, concentrations of Cd grown at this concentration were below the detection limit of the apoplast/symplast assay. Plants were thus grown with a low concentration of Cd^{2+} (1.0 µM) for 28 days and then exposed to an acute, high concentration of Cd^{2+} (5.0 mM) for an hour prior to harvest. Although 5.0 mM Cd^{2+} is a very high concentration and biologically unrealistic for the studied plants, this allowed to measure apoplastic and symplastic Cd separately by chemical desorption of apoplastic Cd^{2+} in $CaCl_2$ solution. It was found that that Cd was evenly distributed between the apoplast and symplast in barley, whereas lettuce stored only 35% of the total root Cd in the symplast and the rest was bound within the apoplast. Whereas lettuce seedlings were wilted at the end of the desorption experiment, indicating Cd stress from acute exposure of Cd, barley seedlings appeared turgid, showing higher ability against Cd toxicity compared to lettuce.

Both PCs and their precursor peptides were synthesized upon Cd exposure in roots of barley. In the case of lettuce, PCs were below the detection limit in the root and only Cys and GSH were detected. The high amounts of PCs and monothiols produced in barley roots could have contributed to the greater Cd-accumulation in barley relative to lettuce as well as to the observed preferential retention of Cd in barley roots (Figure 5.1 F). If Sanità di Toppi and Gabbrielli's (1999) model is correct, after Cd^{2+} is released from a PC complex in the vacuole, the PCs could either be degraded by vacuolar hydrolysis or could return back to the cytoplasm. These apo-PCs could serve as a shuttle, bringing more Cd^{2+} into the vacuole. This shuttling process could continue until all the free Cd^{2+} ions are moved into the vacuole in barley. This could be one of the mechanisms that allowed barley to retain higher concentration of Cd in the root. The fact that no PCs were detected in the roots of lettuce indicates that PCs were not involved in Cd-accumulation in lettuce root and thus most of the Cd^{2+} taken up by lettuce was translocated to the shoot. If lettuce keeps Cd in the apoplast of the root, only a small amount of Cd^{2+} could bind with the pectin present in the cell wall, since only a small amount of pectin would be available in the de-esterified form which can bind Cd^{2+} (Douchiche et al., 2007). The rest of the pectin would be in the methylesterified form, which cannot bind Cd^{2+} under normal conditions. This was supported by the findings from Chapter two and three where a low

concentration of Cd was measured in the root apoplast of lettuce and most of the Cd was translocated to the shoot.

The findings from Chapter three (PC study) were crosschecked in Chapter four, where the distribution of Cd in the roots of lettuce and barley was investigated using multiple imaging techniques, including histochemical staining, scanning electron microscopy assisted with wavelength dispersive spectroscopy (SEM-WDS) and micro-synchrotron X-ray fluorescence (μ-XRF). Altogether, these techniques provided information on the distribution of Cd in the epidermis, cortex, endodermis and vascular bundle and helped to determine the proportion of Cd bound to the cell walls and in the vacuoles. It was found that there were clear differences in the localization of Cd in the roots of lettuce and barley. Whereas the signals of Cd in the apoplast were higher compared to symplast in lettuce, there were no differences between these two compartments in barley. This result supports the findings from Chapter three where apoplastic and symplastic Cd were measured by chemical desorption of apoplastic Cd in $CaCl_2$ solution. At the cellular level, the concentration of Cd was very high in the exodermis (Fig 5.1 B) and endodermis (not shown in Fig 5.1) in the roots of barley, indicating the ability of these tissues to provide barriers against the translocation of Cd towards the stele. On the other hand, while Cd was detected in the roots of lettuce, the concentration was lower compared to barley and there were no differences in the concentrations of Cd among epidermis, cortex, endodermis and vascular bundle, indicating the possibilities of fewer barriers in lettuce root compared to barley.

The reason for the differences between lettuce and barley in providing barriers against Cd could be due to the differential chelation of Cd^{2+} in the cell wall (Figure 5.2 E; in the case of lettuce) and/or inside the cell (Figure 5.1 F; in the case of barley), as well as potential differential efficiency of Casperian band in restricting Cd^{2+} in the exodermis and endodermis (Figures 5.1 C, D, H and I, 5.2 E and F). Since barley had high concentrations of Cd in the exodermis and endodermis, it is possible that fewer Cd^{2+} at the exodermis and endodermis in barley could get past the Casparian bands and accumulated in the cell walls of the exodermis and endodermis.

Once Cd^{2+} crosses the epidermis, exodermis and endodermis barriers and reaches the vascular bundle, Cd^{2+} can again bind to the cell walls of the vascular tissues before loading to the xylem vessels (Isaure et al., 2006; Van Belleghem et al., 2007; Hu et al., 2009). This was observed in Chapter four, where Cd was detected in the vascular tissues of lettuce and barley with the highest concentration of Cd in the cell wall of xylem vessels. The observed differences between barley and lettuce could be explained by the presence of more transporters in the vascular parenchyma of lettuce that could transport Cd^{2+} (Figure 5.2 G and H). Finally, once Cd reaches the shoot, barley might redistribute Cd^{2+} back to the root through a phloem-mediated way (Figure 5.1 K and L) and reduce Cd toxicity in the shoot. Since Cd was not detected in the phloem of lettuce, it is possible that lettuce did not use phloem-mediated Cd^{2+} redistribution from the shoot to the root to mitigate toxic effects of Cd in the shoot.

Lastly, it can be said that although all three species tested in this thesis accumulated Cd upon Cd exposure, they responded differently in terms of Cd distribution in the different organs inside the plant. Among the species, barley accumulated the highest amount of Cd in the root followed by lettuce and radish. The mechanism of higher Cd accumulation in barley compared to lettuce might be related to the ability of barley to retain more Cd in the root by providing barriers in different tissues and also, by immobilizing Cd in the cytoplasm. This knowledge would be useful in designing engineered plants with lower concentrations of Cd in the edible organs. If the results obtained for barley are applicable to other plant taxa, the traits that would be of most benefit in maximizing the retention of Cd in the root include: increased metal-binding components of the cell wall, increased pools of PC precursors in the roots, increase PC-synthase activity in the roots, decreased amounts or expression and activity of metal transporters in the vascular parenchyma and increased expression and activity of transporters that could load Cd^{2+} in the shoot into the phloem for subsequent transport back to the root.

5.2 Future Work

It was deduced from Chapter two that there is a positive relationship between total Cd content and total volume of water transpired per unit leaf area in lettuce, barley and radish, with the strongest correlation in lettuce. Since transpirational water loss is

controlled by stomata, it would be interesting to investigate whether the relationship between Cd accumulation and total volume of water transpired is related to the number of stomata present per unit leaf area and/or the percentage of stomata that are either open or closed due to Cd exposure. Also, since transpiration alone cannot entirely explain Cd accumulation and translocation in the studied plants, another interesting extension of the transpiration study would be to investigate active uptake of Cd^{2+} in plasma membranes of both the endodermis and xylem parenchyma in these species.

In Chapter three, PCs were identified in barley roots and it was assumed that they were available to form complexes with and detoxify Cd^{2+}. However, until Cd^{2+}-PC complexes can be identified in the cellular environment of roots, their role remains theoretical. The problem is that quantification of Cd^{2+} and PCs requires rupture of the cells and dissolution of the cell extract. Thus, any Cd^{2+}-PC complexes that may have been in the tissue are dissociated during sample preparation. One possible approach to finding intact Cd^{2+}-PC complexes in plant tissues could be using micro-synchrotron analytical techniques. Specifically, µ-XANES and µ-XAFS can provide information on element-specific oxidation state(s) and coordination environment(s), respectively, of metals inside the cell. By understanding the neighbouring chemical environments of Cd^{2+} directly in the cellular environment, one can predict which molecule(s) Cd^{2+} is bound to. Another approach could be using HPLC with Mass Spectroscopy (HPLC-MS) to study Cd^{2+}-PC complexes in the cellular extract; however, there is a chance of dissociation of Cd^{2+} during freeze-thawing, extraction and sample preparation for HPLC-MS that makes it hard to measure Cd^{2+}-PC complexes in tissue extracts.

Some studies reported the possibilities of transporters in the vascular bundle that can control plants capacity to load Cd^{2+} from the xylem parenchyma to xylem vessels (Mori et al., 2009; Yamaguchi et al., 2011). In Chapter three, the Cd signal at the cell wall of xylem was higher in barley compared to lettuce. It would be interesting to examine the presence of transporters in the vascular bundle of the roots of lettuce and barley that could be associated with Cd^{2+} loading from the xylem parenchyma to xylem vessels.

Finally since there is a possible redistribution of Cd^{2+} or Cd^{2+}-complexes from the leaves to the sink tissues (stem, root etc.), it would be interesting to investigate whether Cd^{2+} or any form of Cd^{2+}-complexes were present in the phloem saps of lettuce and barley. In the present study, high concentrations of PCs were measured in the shoot tissues of barley compared to lettuce in Chapter three. In Chapter four, a high concentration of Cd was detected in the phloem tissues of barley root, indicating the possibility of redistribution of Cd^{2+}-PC complexes from the shoot to the root. Since the concentrations of PCs were low in lettuce root compared to barley, it is possible that less Cd was redistributed from the shoots to the root in lettuce compared to barley; however, this also needs confirmation. Since the concentrations of Cd in the phloem (and possibly the xylem) might be below the detection limits of ICP, it might be useful to use stable isotopes of Cd^{2+}.

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